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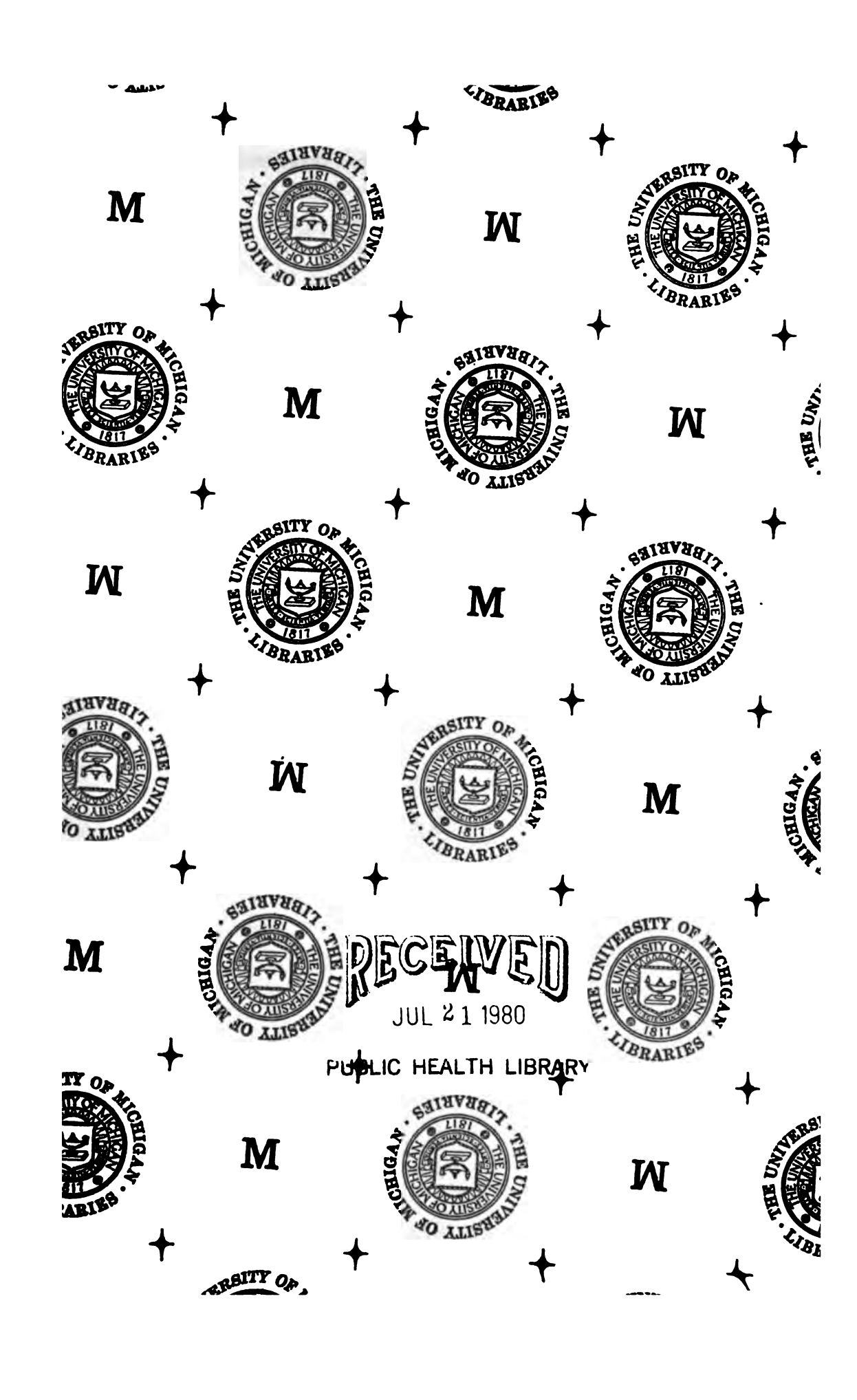
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Volume 162, Number 1, October 1979

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THE FIRST ANNUAL SYMPOSIUM OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE

Atlantic City, New Jersey

April 12, 1978

First Annual Symposium of the Society for Experimental Biology and Medicine, Atlantic City, New Jersey, April 12, 1978

Introductory Remarks[1]

DEWITT STETTEN, JR.[2]

present occasion marks both the 75th
f the Society for Experimental Biology
ledicine and its first national meeting.
3, Dr. Samuel Meltzer, a Russian-born
erman-trained physiologist associated
he Rockefeller Institute, assembled a
of congenial colleagues from the New
medical schools and research institu-
o found a local science club familiarly
ı as the Meltzer-Verein (-Union). It
nonthly scientific meetings, sometimes
how's restaurant or at the Liederkranz
and established a journal, *The Proceed-
f the Society for Experimental Biology
ledicine*, which became its chief asset.
se were the days when travel was ac-
ished predominantly by rail, with a
le horse-drawn vehicle to take the trav-
om the railroad station to his hotel or
ıg place. Many years were to elapse
the concept of a travel award was to
ıe common. In general, scientists met
with fellow scientists and only occa-
ly assembled in national gatherings.
Meltzer-Verein was soon imitated in
ities, and thus there arose some 20-odd
al chapters of the Society for Experi-
l Biology and Medicine. These contin-
meet for varying periods of years, but
el money became more generally avail-
ıd as the airplane replaced the railroad,
al meetings replaced those of strictly
ocieties. As a consequence, when my predecessor, Dr. Dennis Watson, examined the situation two years ago, he found, despite more than 3000 members, only four of the SEBM chapters that were still holding meetings.

When I assumed the office of President of SEBM, I undertook to explore the desirability of this Society holding national meetings. The membership was polled and the overwhelming majority of members supported the notion that we meet together with the Federation of American Societies for Experimental Biology. FASEB proved very receptive to this notion, and there shortly came an invitation from the American Physiological Society to be an institutional guest at the April 1978 meeting in Atlantic City. It was further determined that this meeting should take the form of a one-half-day symposium on a topic of combined medical and basic biomedical science interest. The topic selected was "Diseases of Receptor Sites." I asked Dr. Jesse Roth of the National Institute of Arthritis, Metabolism and Digestive Diseases, NIH, to serve as program chairman and to assemble the roster of speakers. He has, I believe, put together an extraordinarily interesting program, which I note is attended both by members of SEBM and by members of the constituent societies of FASEB. This is precisely what I had hoped to see.

At the conclusion of this meeting, my colleagues on the Council of SEBM will come to a determination as to whether the present experiment is a success and how we may pursue the matter further. I, personally, would look forward to a continuing association between SEBM and FASEB over the years ahead.

---

sented at the Symposium, "Diseases of Receptor held in Atlantic City, New Jersey, on April 12,

sident, Society for Experimental Biology and ne.

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ıGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 3-12 (1979)

An Introduction to Receptors and Receptor Disorders (40608)

ıSE ROTH, MAXINE A. LESNIAK, ROBERT S. BAR, MICHELE MUGGEO, KLARA MEGYESI, LEN C. HARRISON, JEFFREY S. FLIER, HELENA WACHSLICHT-RODBARD, AND PHILLIP GORDEN

ıtes Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

aim of this symposium is to sketch in e the role of receptors in disease states. r than attempting a comprehensive re- we shall present selected examples to ıte broad principles: the role of insulin ors in disorders of glucose tolerance ısulin sensitivity, estrogen receptors in cancer, and acetylcholine receptors in henia gravis. They represent the poly- e hormones, steroid hormones, and ıransmitters, respectively. This choice us to neglect other important applica- ıuch as TSH receptors in Graves' dis- ınd the work on receptors for other ɔptide hormones such as glucagon, TRH, LH-hCG, and prolactin; recep- r other systems such as catecholamines, s, prostaglandins, and lipoproteins; and ors for malaria parasites and viruses, which have their receptors on the cell e. Also excluded have been the roles of gen and thyroid receptors in hormone- nt states and of glucocorticoid and ıteroid receptors in leukemias and other ıant disorders (1–4).

'ogical role of receptors. For a ligand to ɔe its biological effect it must first bind ıpecific receptors on a target cell. The or serves two major functions. First, the or must recognize the biologically ac- oiety by binding it. Second, the com- ɔn of ligand with receptor initiates the ımical events that lead to the biological of the specific ligand system. In the f hormones, the ligand has no function absence of receptor. Whether the re- has function in the absence of hor- (i.e., a basal activity) is as yet unclear. lition to its fundamental roles of rec- ɔn and activation, the ligand–receptor ıtion often participates in other biolog- ınctions. Some of these additional func- tions for the interaction of insulin with its receptor are listed in Table I.

Information transfer. While it is clear *in vivo* that the ligand and cell receptor are both necessary for the system to function, it is worthwhile to consider the relative role of each in transmitting information to the cell (Table II). For some systems, such as sperm and egg, both moieties contribute approximately equal amounts of information. With other systems, the information is in the ligand, and the receptor serves only to concentrate the ligand, process it, and expedite its translocation to its site of action. Thus in the case of low-density lipoproteins (LDL), cholesterol within the LDL molecule is the signal to the cell. The receptor acts to get LDL into the cell where the LDL is processed to yield free cholesterol. If cholesterol is put into the cell by some extra receptor mechanism, the net biological effect is the same. Similarly, toxins such as cholera toxin and diphtheria toxin are enzymes. Binding of toxin to receptor leads to activation of the enzyme and its entry into the cell where the toxin acts. Introduction of the activated toxin by mechanisms that bypass receptor lead to the characteristic effects of the toxins. Thus the signal is entirely within the ligand.

In other cases the full program of information is within the receptor, and the ligand acts solely to cause the receptor to express its program. For example antibodies directed against the receptor for insulin mimic all of the biological effects of insulin acting on the insulin receptor (5, 6). The antibody to receptor (in the absence of insulin) stimulates transport of glucose and amino acids (immediate effects), activation of glycogen synthase and inactivation of phosphorylase (intermediate effects of insulin), and synthesis of lipoprotein lipase (a delayed insulin effect).

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TABLE I. FUNCTIONS OF THE RECEPTOR-HORMONE INTERACTION

A. Fundamental
   1. Recognition
   2. Activation

B. Additional
   1. Reservoir for plasma hormone
   2. Regulate degradation of hormone
   3. Regulate degradation of receptor
   4. Regulate receptor concentration and receptor affinity
   5. Crosslink and translocate hormone-receptor complexes
   6. Regulate postreceptor events

TABLE II. INFORMATION TRANSFER

A. Hormone + receptor $\rightleftarrows$ hormone-receptor complex $\rightarrow$ activation
B. Where is the "*information*" for the activation—H?? R?? HR??
C. Examples of ligand-receptor systems
   1. Both moieties contribute about equally—egg and sperm
   2. Information is in the ligand; receptor acts to concentrate, process, and/or translocate the ligand to an intracellular site
      a. Toxins: cholera, diphtheria, ricin
      b. Low-density lipoproteins
      c. Lysosomal enzymes
      d. Viruses
   3. Information is in the receptor; ligand acts to bring out the program from the receptor
      a. Insulin
      b. IgE
      c. (Thyrotropin)
      d. (Acetylcholine)

That antibodies (and probably concanavalin A), which bind to regions of the receptor outside of the insulin binding site, can initiate the characteristic panoply of insulin effects suggests very strongly that all of the information is in the receptor, and the sole function of insulin is to cause the receptor to express itself. Similar reasoning applies to studies of the IgE receptor where again antibodies to the receptor can replace the normal ligand, which is antigen linked to IgE (7, 8). Though less well studied in this regard, two other examples where the receptor appears to contain the fundamental information are the receptors for acetylcholine and for TSH.

Studies of this type which dissect the role of ligand and receptor in the process of information transfer are important in understanding the mechanism of disease and potentially have important therapeutic consequences. For example, in patients who are insulin deficient, the current therapeutic strategy is now focused on improved methods of insulin delivery such as islet-cell transplantation and automated systems for insulin infusion. If the receptor has all of the information, then the hormone can be bypassed and a much broader range of agents that act on receptor can be surveyed as potential substitutes for insulin.

Receptors in endocrine systems. As shown in Fig. 1, hormone released from the gland travels in the plasma to target cells throughout the body where it binds rapidly to a finite number of receptors on the cell. Hormones that are lipid soluble such as iodothyronines, steroids, and vitamin D derivatives quickly (freely?) cross the plasma membrane. Their receptors are inside the cell, and their primary site of action is in the nucleus (Table III).

The peptide hormones, which represent at least 80% of all hormones, and the catecholamines are water soluble and do not easily cross the lipid barrier posed by the plasma membrane of the cell. Their specific binding sites are on the outer surface of the plasma membrane (9) (Table III). The receptors which contain the specific binding sites are integral membrane proteins (which cannot be freed from the membrane except by the use of detergents). The combination of receptor with hormone generates a transmembrane message, which leads to the activation of

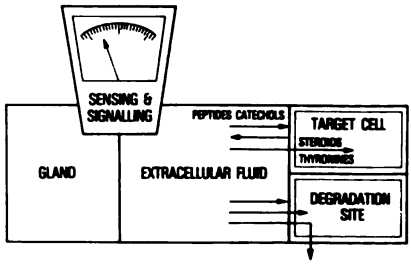
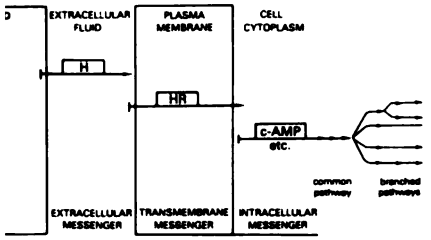


FIG. 1. Schematic diagram of an endocrine system. Sensing device(s) signals the gland to release hormone into the extracellular fluid in which it travels to reach cells throughout the body. Hormones that are lipid soluble such as steroids, 1,25$(OH)_2$-vitamin D_3, and iodothyronines traverse the plasma membrane of the cell and interact with receptors that are intracellular. The peptide and catecholamine hormones, which are water soluble and do not steadily traverse the lipid barrier of the cell membrane, interact with their receptors on the outer surface of the cell.

TABLE III. CLASSIFICATION OF HORMONES

Chemical class	Percentage of all hormones	Solubility	Receptors	Postreceptor
'eptides	≧80	Polar	Cell surface	Second messenger
:atecholamines	≦5			
iteroids (and sterol)	≦15	Nonpolar	Intracellular	Nucleus
odothyronines	≦5			



2. Cellular events in the action of peptide and lamine hormones. The hormone (H) is the extra- messenger, carrying the signal from the gland to :r surface of the target cell, where it combines ecific receptors (R) to form hormone–receptor tes (HR). The latter initiates a transmembrane ; the nature and form of the transmembrane ;er are unknown. The next step for many of these es is activation of adenylate cyclase at the inner of the plasma membrane, which generates cyclic :AMP), a soluble intracellular messenger that s specific protein kinase(s). This sequence of lular events is designated "common pathway." ase is thought to activate and inactivate many lular proteins, which leads to the many biological haracteristic of a given hormone in a single target signated "branched pathways." For hormones not activate adenylate cyclase, the "common /" is unknown but thought to be of similar gen- ign.

:llular processes (Fig. 2). The nature of ransmembrane messenger(s) is not ı. The earliest known intracellular for many peptide hormones and for β- :rgic catecholamines is activation of late cyclase, followed by production of AMP and activation of cyclic AMP- dent protein kinase(s). All (or most) of :ects of these hormones are thought to result of action of the specific kinase(s). ormones such as insulin, prolactin, ı hormone, and the α-adrenergic cate- nines that do not act via the adenylate :–cAMP kinase pathway, the nature of :ly intracellular events is unknown, but >een widely assumed that the design of ıtem is similar.

Many receptors for nonendocrine systems (neurotransmitters, LDL, prostaglandins) are membrane proteins with binding sites on the cell surface and are similar in many respects to receptors for peptide and catecholamine hormones. Exceptions thus far are receptors for cholera toxin which appear to be GM_1 gangliosides on the cell surface and possibly receptors for other microbial products or agents.

Quantitative aspects of the hormone–receptor interaction. Free hormone (H) in the plasma or extracellular fluid binds reversibly to receptors (R) to form hormone–receptor complexes (HR) (Table IV). The concentration of these complexes, [HR], determines the magnitude of the signal to the cell. The biological effect is some function, f, of the concentration of HR, which in turn is a function of hormone concentration [H], receptor concentration [R], and the affinity of receptor for hormone, K. Since all three ([H], [R], and K) are partners in determining the signal rate to the cell, the signal will vary in response to changes in any of the three determinants (Table IV). While it has been clear that hormone concentrations fluctuate rapidly *in vivo*, it has become clear only recently that receptor concentration and affinity both fluctuate in response to biologically relevant stimuli and that these wide and often rapid responses contribute greatly in determining the magnitude of the biological response observed.

Biologically relevant fluctuations in receptor affinity and concentrations. Historically pharmacologists and endocrinologists, in their use of biological systems, often selected conditions to minimize or obscure changes in target cell sensitivity including changes in the receptor. Thus the pharmacologists who wished to study agonists and antagonists wished to have the biological response that they observed reflect only the effect of alterations in the nature of the ligand or its concentration. Similarly, the endocrinologists in their study of

TABLE IV. THE INTERACTION OF HORMONE WITH RECEPTOR

1. The primary interaction

$$H + R \rightleftarrows HR$$

H = free hormone
R = free receptor
HR = hormone–receptor complexes
R_o = total receptors (R + HR)

2. The equilibrium constant (K_a) for this reaction

$$K_a = \frac{[HR]}{[H]\,[R]}$$

3. The biological effect (E) is some function (f) of the strength of the signal to the cell; the signal strength is directly related to [HR].

$$E = f([HR])$$
$$= f(K[H][R])$$
$$= f\left(\frac{K[H][R_o]}{1 + K[H]}\right)$$

hormones set conditions so that the biological response they observed reflected only changes in the concentration of hormone. Thus bioassays for drugs, hormones, and other ligands depend entirely on the ability of the investigator to fix the responsiveness of the target cell. That so few bioassays work well and require such meticulous attention to methodological detail attest to the fact that target cells by their nature are not fixed targets but rather change their responsiveness rapidly in response to numerous stimuli. Given the natural propensity of target cells to regulate their responsiveness so quickly and so widely makes it clear, retrospectively at least, why bioassays require such herculean efforts to make them perform adequately.

While any biochemical step within the target cell can be responsible for the changes in responsiveness, recent studies make it clear that the first step, the binding of ligand to receptor, is frequently affected (10–19). In the case of the receptor for insulin, numerous biologically relevant events produce changes in the receptor (Table V). Growth rate, differentiation, and environmental conditions can have profound effects on the receptor. Dietary composition, total calories, timing of meals, and exercise have major effects at the level of the receptor concentration or affinity. The homologous ligand itself acts to regulate both concentration and affinity of its own receptor; regulation of receptor concentration is a regulatory event that requires cellular events beyond the receptor whereas regulation of receptor affinity appears to be a physicochemical process intrinsic to the receptor that can be reproduced fully with detergent-solubilized receptors. Other (heterologous) hormones and drugs can affect target cell receptors for insulin and thereby alter target cell sensitivity to insulin. A condensed list of these regulators is presented in Table V. Given that the receptor is at the interface between the inside and outside of the cell, it is reasonable retrospectively that it is responsive to signals from both the external and internal environments of the cell.

TABLE V. BIOLOGICALLY RELEVANT REGULATORS OF AFFINITY AND/OR CONCENTRATION OF INSULIN RECEPTORS

1. Exercise	7. Age
2. Meals	8. cAMP
3. Diet	9. pH
4. Insulin	10. Ketones
5. Other hormones	11. Sulfonylureas
6. Growth and development	12. Anti-receptor antibodies

Historical perspective. The concepts of hormones and of receptors were both introduced in the last quarter of the 19th century and were clearly formulated during the first decade of this century. During the next 60 to 70 years the study of hormones advanced rapidly while that of receptors lagged. The natural advantages of hormones, some of which are cited in Table VI, explain some of this disparity in progress. Basically, the hormone was easier to study.

The dominance of hormone over receptor (and target cells) has been so great that endocrinology, instead of being the study of

ILE VI. NATURAL FACTORS THAT FAVORED PROGRESS WITH HORMONES

	Peptide hormones	Cell surface receptors
concentrated in a lo- d region	Yes	No
in aqueous solvents	Yes	No
cal effect when intro- d *in vivo* and *in vitro*	Yes	No
in blood	Yes	No

rine systems, has been viewed as the of hormones. Likewise endocrine dis- have been viewed as diseases of hor-. Clearly if an endocrine system is de-d, any one of the steps can be at fault. ost disorders have been viewed simply to hormone excess or hormone defi-.

en the biological effect of a hormone deficient, hormone deficiency was d. Only later did it become clear that ny disorders hormone was present at l or supernormal concentrations and he target cell was defective. Thus hy-emia due to parathormone deficiency cognized long before target cell resist-to the hormone (pseudohypoparathy-m); polyuria due to vasopressin defi- before vasopressin-resistant states (ne-enic diabetes insipidus); dwarfism due wth hormone deficiency before growth one-resistant (Laron-type) dwarfism; ailure of sexual development due to gen deficiency before the hormone-re- causes. In the examples cited, resist-o the hormone was complete or almost ete. In diseases where the effectiveness hormone was reduced but not absent, nition of the defects were even slower to . Thus with insulin resistance, the sen- of the target cell to insulin is reduced ot totally absent and therefore more . (Total refractoriness to insulin may be

orders of glucose metabolism. It has long known that glucose stimulates the β-f the pancreas to release insulin which n target cells to promote glucose utili- and storage. However, only glucose be measured so that the system was nplified—hyperglycemia was due to deficiency and hypoglycemia was due to insulin excess. The dramatic response of diabetics to insulin administration and the similarity of patients with islet-cell tumors to symptoms of diabetics who received too much insulin strengthened this simple formulation. With the introduction of methods to measure insulin levels in blood, especially the radioimmunoassay, it became clear that only a minority of patients conform to this scheme (13). The diabetics who are truly insulin requiring are indeed insulin deficient but the majority of hyperglycemic patients are not absolutely insulin dependent and have normal or supernormal concentrations of circulating insulin (Table VII). Further, the insulin in these patients is biologically intact insulin. Conversely in patients with hypoglycemia due to islet-cell tumors, insulin levels were found to be inappropriately elevated, which conformed to expectations, while in many patients with low normal glucose or frank hypoglycemia, plasma insulin was noted to be low or unmeasurable (Table VII). Thus the radioimmunoassay for insulin, while it

TABLE VII. CONCORDANCE AND DISCORDANCE OF CIRCULATING HORMONE CONCENTRATIONS AND THE BIOLOGICAL STATE

	Hormone concentration subnormal or unmeasurably low	Hormone concentration normal or elevated
Biological response deficient	Concordant Insulin-dependent (juvenile type) diabetes (~20%)	Discordant Non-insulin-dependent (adult-type) diabetics (~80%) Growth hormone excess Glucocorticoid excess Uremia Syndromes of extreme insulin resistance
Biological response excessive	Discordant Non-islet-cell tumors Anorexia nervosa Growth hormone deficiency Glucocorticoid deficiency	Concordant Islet-cell tumors Infants of diabetic mothers

confirmed that hormone levels were concordant with biological observations in some patients, raised a paradox in most conditions—the hormone levels were discordant with the biological observations (Table VII). The introduction of methods to measure the receptor has resolved many of these apparent paradoxes. It was shown in many disorders of glucose metabolism, where hormone levels were discordant with the clinical state, that the receptor was altered and reflected the clinical state.

Receptor methods. The first attempts to measure receptor, reported 30 years ago, failed because it was not clear that the labeled hormone was biologically active, degradation of hormone and of receptor were not controlled, and it could not be shown that the observed binding of hormone to tissue was biologically relevant, i.e., nonspecific binding accounted for most or all of the binding (14, 15). Subsequent studies of this type suffered the same shortcomings.

The modern era of receptors began in the sixties when studies were aimed at overcoming these problems. For steroid hormones, the problem was somewhat simpler (16, 17). Tritiated hormones, prepared for other studies, were bioactive and therefore suitable for receptor studies. The receptors were soluble cytoplasmic components and therefore the well-developed techniques for studying the binding of two soluble components were applicable. However these methods were not applicable to the vast majority of hormones or to most nonendocrine receptor systems; the radioactive ligands available were unsuitable (i.e., of uncertain biological activity or inadequate specific activity), and the methods for dealing with membrane-bound proteins (receptors) were primitive.

The current methods for studying cell-surface receptors are based on the methods introduced for the study of ACTH and angiotensin binding to their specific receptors (18–22). The approach of the two pioneer groups were similar. Meticulous attention was given to preparing radioactive ligand at very high specific activity that retained biological activity. The receptor preparations were handled to minimize both nonspecific binding and degradation of hormone and receptor. Finally the binding of labeled hormone was shown to have the precise specificity of the biological system, i.e., bioactive analogs competed for binding in proportion to their biological potency, and irrelevant materials (e.g., other substances including other hormones) had no effect. These techniques have proved widely applicable to all other peptide hormones and catecholamines, as well as to neurotransmitters including the opiate-like agents, prostaglandins, lectins, toxins, lipoproteins, microbes, and other agents that have cell surface receptors (Table VIII).

Receptors and disease. With the introduction of methods to study receptors directly, most studies were focused on biochemical characterization of the systems. Breast cancer and leukemia as well as nonmalignant disorders were the first diseases where receptors were implicated as having an important role (22–27). Bullock and Bardin noted that an androgen-resistant state, testicular feminization in one strain of mice, is associated with a severe deficiency of androgen receptors (24). In the same year Kahn *et al.* found that insulin resistance in mice associated with hyperinsulinemia and obesity is characterized by a deficiency of insulin receptors (25). Since that time a wide range of androgen-resistant and insulin-resistant states in humans and in animals have been shown to be associated with receptor defects (6, 24–33). Further, the severity of the hormone resistance often correlated well with the severity of the receptor defect. Several treatments that improved the defects in insulin responsiveness were associated with improvements in the receptor. Conversely, heightened sensitivity to insulin and/or insulin deficiency were associated with increased binding of hormone to receptor, and correction of the clinical disorder correlated with correction of the receptor abnormality. Since that time, receptor defects have been found in other areas of endocrinology as well as in other nonendocrine re-

TABLE VIII. CELL SURFACE RECEPTORS

ACTH and *Angiotensin* → Other H →	Other surface receptors
Oxytocin	Toxins
Glucagon	Lectins
Insulin	Neurotransmitters
Growth hormone	Opiates
Gonadotropins	LDL
Catecholamines	Prostaglandins
Other	Other

/stems, and many disease mecha- ıt were poorly understood have be- ıch clearer (31–37).

'tative versus qualitative defects. In orders, the receptor is absent or so deficient that the biological effect of d is virtually nil (Table IX). Some of ıone-resistant states, including some -resistant malignancies, the homo- ›rm of familial hypercholesterolemia the resistance to one form of malaria ıples of this class (35). In other dis- ıe receptor is present but the concen- r affinity of receptor is altered so that t of a normal concentration of the roduces a deficient or excessive re- The mechanisms for these alterations :rous. Examples include most disor- ınsulin responsiveness, other endo- ıormalities, and the most common 'amilial hypercholesterolemia.

ther group of disorders the mecha- : more complex and involve a dys- of the receptor (Table IX). In a rare familial hypercholesterolemia, the can bind the ligand but the receptor ive; it does not concentrate in the its on the cell surface and bound not internalized. In Graves' disease, :s to the TSH receptor cause the receptor to signal the cell in the absence of the normal ligand and without regard to normal control mechanisms (36). In myasthenia gravis, the antibody to the acetylcholine receptor alters receptor turnover and function (37). In several forms of insulin resistance antibodies to receptor not only impair the affinity of receptor for ligand but can also acutely mimic insulin action and can also chronically impair the sensitivity of the target cell to insulin at some site beyond binding of hormone to receptor. When the defect in the receptor is more than just a defect in binding, it is more difficult to detect and to unravel.

In addition to the situations where the receptor is abnormal quantitatively or qualitatively, there is a group of disorders due to defects in the nature of receptor design (Table X).

Disorders due to defects in receptor design (31–33). Ideally, each hormone should be a unique entity and have a unique set of receptors. This would confer absolute specificity on the system (Fig. 3, "Ideal"). In many cases the hormone is not truly unique chemically or in its binding to receptors; there is some limited degeneracy built into the system. Thus one hormone, in addition to its strong reactivity with its own receptors, may also react weakly (i.e., with much lower affinity)

BLE IX. EXAMPLES OF DISEASES AND OTHER RELEVANT CONDITIONS RELATED TO RECEPTORS

ıbsence of ligand binding to receptor
›gen-insensitive states—several
ne insulin resistance in one case of leprachaunism
disorders with total refractoriness to hormone
receptors in homozygotes with familial hypercholesterolemia
-negative human erythrocytes are resistant to vivax malaria
ed binding of ligand to receptor
n-resistant states—many
receptors in heterozygotes with familial hypercholesterolemia
holamine receptors in asthmatics who receive adrenergic agents chronically
:d binding of ligand to receptor
tened responsiveness to insulin in anorexia nervosa, growth hormone deficiency, glucocorticoid deficiency
id hormone increases catecholamine receptors
;ens increase oxytocin receptors
;ens increase progesterone receptors
x disorders involving receptors
›dies to TSH receptor in Graves' disease mimic TSH and cause hyperthyroidism
›dies to acetylcholine receptor are responsible for myasthenia gravis
›dies to insulin receptor in Type B extreme insulin resistance, ataxia telangiectasia, other immune ciency states, NZO mice
tive LDL receptor in one patient with familial hypercholesterolemia
rs of receptor design (specificity spillover)—see Table X

TABLE X. CANDIDATES AS DISORDERS POSSIBLY DUE TO DEFECTS IN RECEPTOR DESIGN—SPECIFICITY SPILLOVER

Clinical condition	Hormone in excess	Reacts with receptors for	To produce
Non-islet-cell tumors	NSILA-s (IGF-II)	Insulin	Hypoglycemia
Babies of diabetic mothers	Insulin	Insulin-like growth factors	Macrosomia
Untreated Addison's disease; Autonomous overproduction	ACTH	α-MSH	Skin darkening
Acromegaly	hGH	Prolactin	Galactorrhea, amenorrhea
Choriocarcinoma	hCG	TSH	Hyperthyroidism
Primary hypothyroidism in childhood	TSH	LH, FSH	Precocious puberty
Glucocorticoid excess	Hydrocortisone	Aldosterone	Hypertension
Hypertension of pregnancy; Essential hypertension	Oxytocin? Other nonapeptide?	Nonapeptide pressor	Hypertension

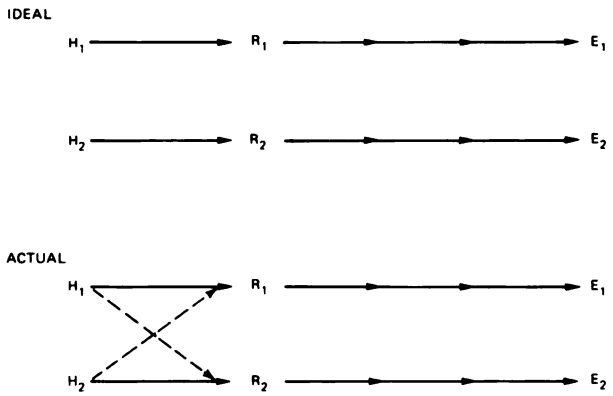
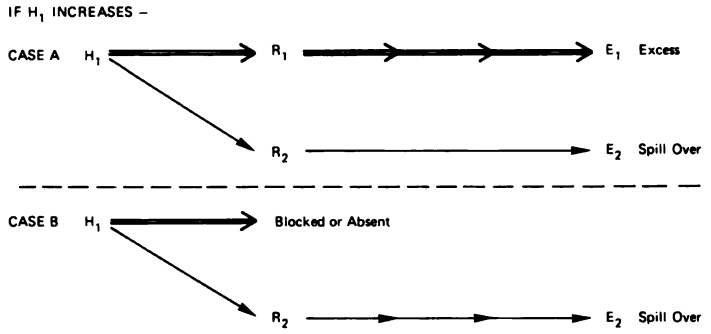


FIG. 3. Specificity in hormone–receptor interactions. In the ideal case, each hormone (H) has its own unique receptor (R) with which it combines to produce its specific series of biological effects (E) and has no affinity at all for receptors of other hormones. Actually one hormone (H_1), in addition to its strong reactivity (→) with its own receptors (R_1), can react with a weak but finite affinity (⟶) with receptors (R_2) of a closely related hormone (H_2); high concentrations of H_1 can thereby produce its own characteristic effects (E_1) as well as those effects (E_2) characteristic of the related hormone (H_2).

with receptors of a closely related hormone (Fig. 3, "Actual"). Under ordinary circumstances the weak reactivity of a hormone for receptors of another may be of no consequence. However, when the concentration of the hormone is increased (as in states of hormone excess) the hormone may now act to stimulate this second pathway (i.e., to produce the biological response characteristic of the other hormone), in addition to stimulation of its own pathway (Fig. 4). The cross reaction of one hormone onto the receptor of another is not random (i.e., not "promiscuous") but each occurs only within the bounds of a well-defined family of closely related hormones and receptors (i.e., "incestuous").

Table X contains a list of diseases characterized by hormone excess in which one or more symptoms may be due to one hormone acting to stimulate the receptor for another (related) hormone (31–33). We have designated these kinds of disease manifestations as due to specificity spillover at the receptor. It should be emphasized that this general mechanism has not been proven; rather we have proposed this as a possible disease mechanism to provide a simple explanation for some unexpected manifestations of hormone excess. The validity of the concept can be tested experimentally. These suggested examples would add to the list of disorders where examination of a receptor provides a major advance in our understanding of disease process.

Summary. In the last decade reliable methods have been introduced to quantitate and characterize receptors for hormones and



. 4. Specificity spillover at the receptor. In case A, the concentration of one hormone (H_1) is increased. gh its interaction with its own receptor (R_1) it produces an excess (⟹) of its characteristic effects (E_1), and h its interaction with the receptors (R_2) of a related hormone, it produces effects (E_2) characteristic of the other ne (H_2). In case B, the concentration of H_1 is increased but its own pathway of action is blocked or absent and ly biological effects (E_2) are those produced by the interaction of H_1 with R_2.

biologically interesting ligands. Applied lly to hormone receptors on malignant and to androgen-resistant and insulin- ant states, these methods have led to the ification of many disease processes e the receptor plays an important role. includes not only endocrine-related dis- but also neurological, metabolic, infec- and immune disorders as well.

e. On the basis of biological and chemical data, it 's that the 40 to 50 different hormones found in a organism each evolved from a much smaller num- primordial hormones. Presumably, for the peptide nes the gene for the primordial hormone was ated as was the gene for its cell surface receptor; w hormone–receptor pair evolved to establish a l signaling system. The affinity of each hormone specific receptor was high but in many cases the retained some finite (albeit low) affinity for the eceptor. Thus, teleologically, greater diversity was or by a small loss in specificity. The weak affinity : hormone for the receptor of a closely related ne forms the basis for specificity spillover. The t impairment of specificity in endocrine systems, tulated origins, and suggested consequences may ignificant counterparts in other areas of biology diversity coexists with modest degeneracies in city, e.g., the large series of trypsin-like proteases gulate multiple extracellular events such as clot- id kinin generation; neurotransmitters and related cyclase and the nucleotide-stimulated kinases; ıtibodies and prostaglandins.

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Role of Insulin Receptors and Receptor Antibodies in States of Altered Insulin Action (40609)

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first step in the action of insulin is ; to a surface membrane glycoprotein ır (1). This receptor serves two func- he first is to recognize insulin from all ubstances in blood and bind it tightly. , this interaction of the hormone with eptor then generates some form of embrane signal which initiates a series hemical pathways that ultimately re- production of insulin's biological ef- lthough the insulin receptor has not n purified, over the past 8 years, stud- e been conducted using radioactively insulin which have given some insight e nature of the receptor and the bio- al characteristics of its interaction with . One of the most important observa- erived from these studies has been that eptor is not a static entity—rather it altered in its number or affinity for . Since the receptor is the crucial link lin action at the cellular level, such ons play an important role in the path- is of a number of disease states.

acteristics of the insulin receptor. As above, the insulin receptor has not yet urified and thus most of our informa- out its structure is indirect. A sche- nodel of the receptor is shown in Fig. receptor is an integral membrane pro- id can be solubilized in active form neutral detergents (2–4). The receptor is carbohydrate, although there is no e that this carbohydrate is important lin binding (3). In Triton X-100, the r behaves as a molecule with a Stokes of 70 Å, although several recent studies that this large complex contains sev- units or closely associated proteins (2, stimates for the size of the insulin ; site itself range from 90,000 to) (5, 8–10).

interaction of insulin with its receptor lex and has been the subject of much controversy. Scatchard analysis of insulin binding data results in a curvilinear plot (Fig. 2) consistent with multiple classes of binding sites or negative cooperativity among receptors (11, 12). Using kinetic experiments, as well as equilibrium experiments with a variety of insulin analogues, De Meyts *et al.* (12) have presented convincing evidence for negative cooperativity in insulin binding, i.e., the affinity of the receptor decreases with increasing occupancy. Although not all workers agree on this point (3, 13), we have found this model useful for data analysis in disease states, since it allows for easy comparison between different groups or individuals.

In the cooperative model (assuming an otherwise homogeneous class of binding sites), the Scatchard plot can be viewed as being formed by a family of lines of decreasing slope originating from the various points of the curve and terminating at the intercept of the plot on the abcissa (Fig. 2) (14). The Scatchard plot can then be described by three parameters: (i) The total receptor concentration (R_0) which is the intercept of the plot on the abcissa; (ii) the limiting high-affinity state of the receptor which is observed as occupancy approaches zero (this has been termed $\bar{K}_e$ or affinity of the empty receptor); and (iii) the limiting low-affinity state of the receptor which is observed as occupancy approaches 100% (this has been termed $\bar{K}_f$ or the affinity of the filled receptor). An even more detailed description of receptor affinity may be had by plotting the changing affinity as a function of occupancy in what has been termed an average affinity profile (Fig. 2, right) (14).

While quantitative analysis of insulin binding data has lead to certain models of insulin action and has been useful for descriptive purposes in disease, it is important to keep in mind several important limitations of this approach. First, whether one chooses the cooperative model or the two-site model, these

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are *only models*, not proven mechanisms, of the insulin–receptor interaction. Second, they are derived analyses which are only as good as the original data from which they were taken. In view of the complex nature of the insulin–receptor interaction, it is not surprising that too often these analyses are made on inadequate data. Even with computer-assisted data analysis and multiple data points, the errors in estimation of binding capacity and affinity are large (15)—much larger than most investigators realize or admit. Finally, it is important to keep in mind the fact that virtually all of the receptor studies are done *in vitro* under conditions designed to minimize the complexity of the binding interaction. At 37°, *in vivo*, there is a considerable number of complicating reactions, including internalization (16–18) and degradation (11, 16) of hormone, which may invalidate all of these *in vitro* analyses.

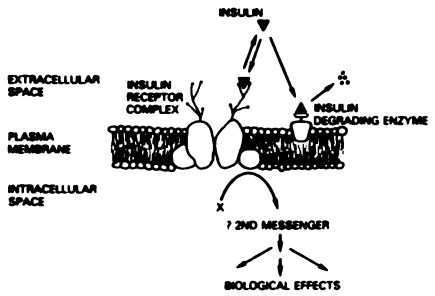


FIG. 1. A schematic model of the insulin receptor. The receptor is viewed as an integral membrane glycoprotein, containing more than one binding site and several subunits. The receptor is chemically distinct from the insulin degrading enzymes.

Obesity as a model of disease with altered receptor concentration. The most common form of insulin resistance in man and animals is that associated with obesity. The insulin resistance of obesity is characterized by hyperinsulinemia, variable degrees of glucose intolerance, and resistance to both endogenous and exogenous insulin (19). The major defect appears to be at the level of the target cell, since the circulating insulin and proinsulin-like materials are normal, and the target tissues demonstrate the same type of subnormal response when challenged with exogenous insulin or when studied *in vitro*. The role of the insulin receptors in disease was first illucidated in studies of obesity, in particular, the obese hyperglycemic mouse (20, 21).

The obese hyperglycemic mouse is an autosomal recessive trait (*ob/ob*) characterized by hyperphagia, excessive deposition of fat, hyperglycemia, hyperinsulinemia, and pancreatic islet cell hyperplasia. These animals have a marked resistance to both endogenous and exogenous insulin, both *in vivo* and *in*

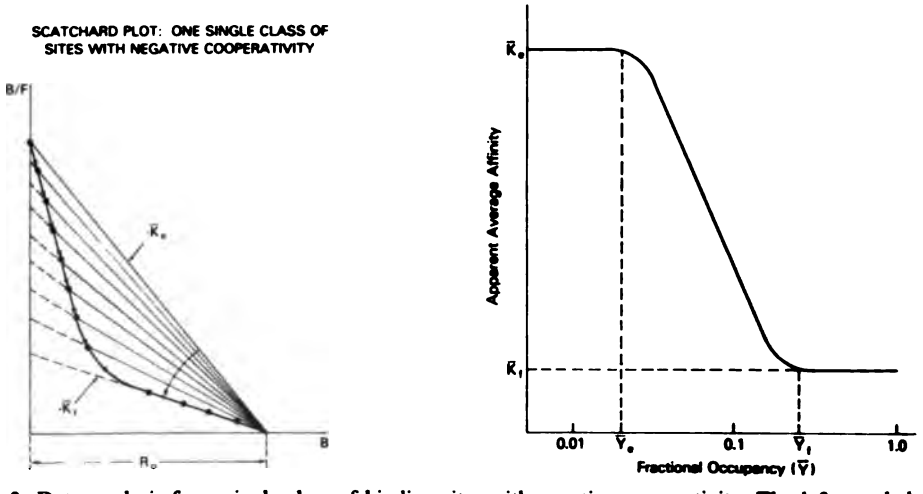


FIG. 2. Data analysis for a single class of binding sites with negative cooperativity. The left panel shows the curvilinear Scatchard plot obtained by plotting the bound/free ratio for insulin (B/F) versus the amount bound (B). R_0 is the total receptor concentration; $\bar{K}_e$ is the affinity of the high-affinity state (empty receptor); and $\bar{K}_f$ is the affinity of the low-affinity state (filled receptor). The right panel shows an average affinity probe obtained by plotting $\bar{K}$ as a function of fractional occupancy ($\bar{Y}$). Adapted from Ref. (14).

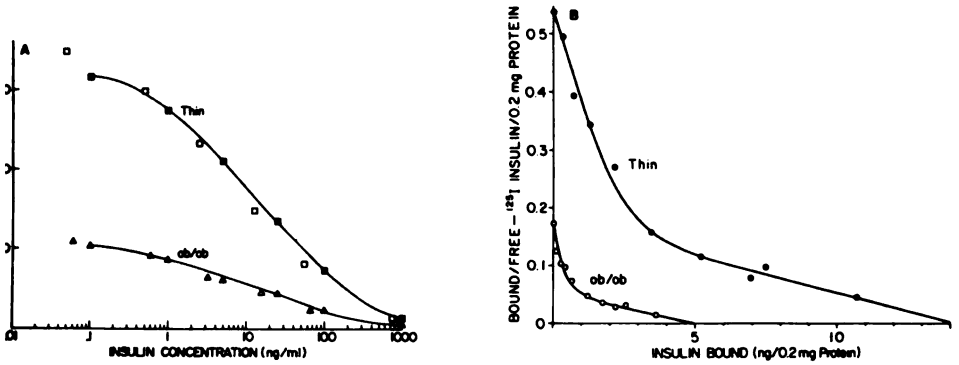
When liver membranes of the *ob/ob* hin mice were prepared and incubated r identical conditions, the binding of n to the membranes of the obese mice onsistently found to be 25–35% of that ved in the thin animals (Fig. 3A) (20). decrease in insulin binding was not ıe to liver, but was also observed in ıs using adipocyte membrane (21), my- ial muscle membranes (22), and intact ic lymphocytes (23).

e difference in observed binding appears due to only a decrease in the number of tors (20, 24). When the bound/free of ıbeled hormone is expressed as a func- ıf the bound hormone (Scatchard plot), ically shaped curves are obtained (Fig. The decrease in insulin binding observed se mice can be accounted for completely decrease in R_0 determined from the ept of the Scatchard plot, whether one es to analyze the data using a coopera- r multisite model.

ce that time, a variety of animals models esity, including human obesity, have studied (Table I) (25–31). These include ıls with acquired and genetic obesity autosomal recessive and polygenic), an- with hyperplastic and hypertrophic y, and animals in which the obesity is ated with normal or abnormal glucose nce. With the exception of the Zucker at (28), cells from each of these animals decreased insulin binding to membrane tors in the basal state. In each case, the ase in insulin binding can be fully ac- ed for by a decrease in the number of n receptors. The receptors which remain are normal in their affinity for insulin, kinetics of association and dissociation, temperature dependence of binding, biological specificity, and site–site negatively cooperative interactions (24).

The major factor regulating the receptor in each case appears to be the basal level of circulating insulin. Thus, the more insulin resistant the animal, the higher the basal insulin and the greater the decrease in receptor number (Table I). Correction of the hyperinsulinemia by diet (31), streptozotocin treatment (21), or diazoxide therapy (32) for only a few weeks in man or a few days with rodents results in correction of the receptor defect, even though significant obesity persists.

The concept that insulin may directly effect the concentration of its own receptors has been substantiated by both *in vitro* studies and experimentally induced hyperinsulinemia *in vivo*. Cells cultured in the presence of increasing concentrations of insulin showed a time-dependent loss of insulin receptors reaching a new steady state in 4–16 hr (33, 34). The steady-state level of receptor is a function of insulin concentration. Similarly, in patients with insulinoma (35) and in rats made chronically hyperinsulinemic by injection of exogenous insulin (oral glucose being given to prevent hypoglycemia) (36), there is a time and dose-dependent decrease in the number of insulin receptors. While the exact mechanism by which insulin regulates its receptor remains unknown, it has been suggested that chronic exposure of cells to high insulin concentrations induces an acceleration in the rate of receptor degradation (37).



3. (A) Insulin binding to purified rat liver plasma membranes of *ob/ob* mice and their thin littermates. (B) ırd analysis of these data. Reprinted with permission from Ref. (20).

TABLE I. CHARACTERISTICS OF INSULIN RECEPTOR FUNCTION IN OBESE ANIMALS

Animal Model	Inheritance	Adipocyte hyperplasia	Glucose intolerance	Increased IRI[a]	Decr rece
ob/ob mouse	Autosomal recessive	+	++	+++	++
db/db	Autosomal recessive	−	+++	+++	++
NZO mouse	Polygenic	+	+	++	++
KK mouse	Polygenic	?	+	+	+
Zucker rat	Autosomal recessive	+	±	+	±
Gold thioglucose-treated mouse	Acquired	−	±	0 to ++	0 to
Man	?	+ or −	0 to ++	0 to ++	0 to

[a] IRI, immunoreactive insulin.

Even normal physiologic concentrations of insulin appear to induce a mild regulatory effect on receptor number. Thus, insulin receptors appear to show a small, but significant, diurnal variation due to changes in insulin level (38) and are significantly increased in hypoinsulinemic, diabetic animals (39). Other diseases with altered receptor concentration are summarized in Table I.

Glucocorticoid excess as a model of disease with altered receptor affinity. It has been well known that when injected into animals under appropriate conditions ACTH and glucocorticoids can produce a form of insulin-resistant diabetes (40). The clinical correlates of these hormonally induced forms of glucose intolerance are also observed in patients with Cushing's syndrome or patients given glucocorticoids for therapy of other diseases (41). Conversely, in the absence of ACTH or glucocorticoids, there is an increase in insulin sensitivity and a tendency toward hypoglycemia (42). The most extreme example of this type of insulin resistance occurs in rats bearing the MtT transplantable pituitary tumor that secretes ACTH, growth hormone, and prolactin. Goldfine and co-workers have shown that in these animals there is a significant reduction in insulin binding to liver membranes, while glucagon remains unchanges (43). This decrease in binding can be corrected by adrenalectomy despite continued high circulating levels of growth hormone and ACTH. More recently, Olefsky *et al.* (44) and Kahn *et al.* (15) have shown that normal rats given high doses of ACTH or dexamethasone show a similar decrease in binding (Fig. 4).

The exact cause for the decrease in insulin binding was initially difficult to ascertain. However, using the negative cooperativity model and computer-assisted data analysis we found that, in contrast to the decr binding observed in obesity, this decr binding seems to be due to a marked d in the affinity of the insulin recep insulin (15). The changes on both K_a are more or less proportional and the a profiles are parallel, suggesting that n cooperativity is conserved (Fig. 4). versely, adrenalectomy produces an i in insulin binding by increasing the a of the insulin receptor, consistent w increase in insulin sensitivity that oc this state. At present, we have not per sufficient kinetic studies to decide i changes in affinity are due to changes association or dissociation rate const is worth pointing out that without the a number of adequate data points a computer analysis, it would have be possible to determine the nature of the tor defect. Also, in contrast to obesit likely that there are significant char intracellular metabolism beyond the r which contribute to the insulin resistan 46). Other disorders with major alterat receptor affinity are summarized in T

Antibodies to the insulin receptor in resistant states. From the preceding sions, it is clear that insulin action

TABLE II. DISEASES ASSOCIATED WITH CHAN THE CONCENTRATION OF THE INSULIN RECE

Receptor concentration decreased
—Obesity
—Thin, hyperinsulinemic diabetics
—Growth hormone excess
—Uremia
—Insulinoma
—Syndrome of insulin resistance and acanth gricans Type A
Receptor concentration increased
—Hypoinsulinemic diabetes
—Hypophysectomy (GH deficiency)

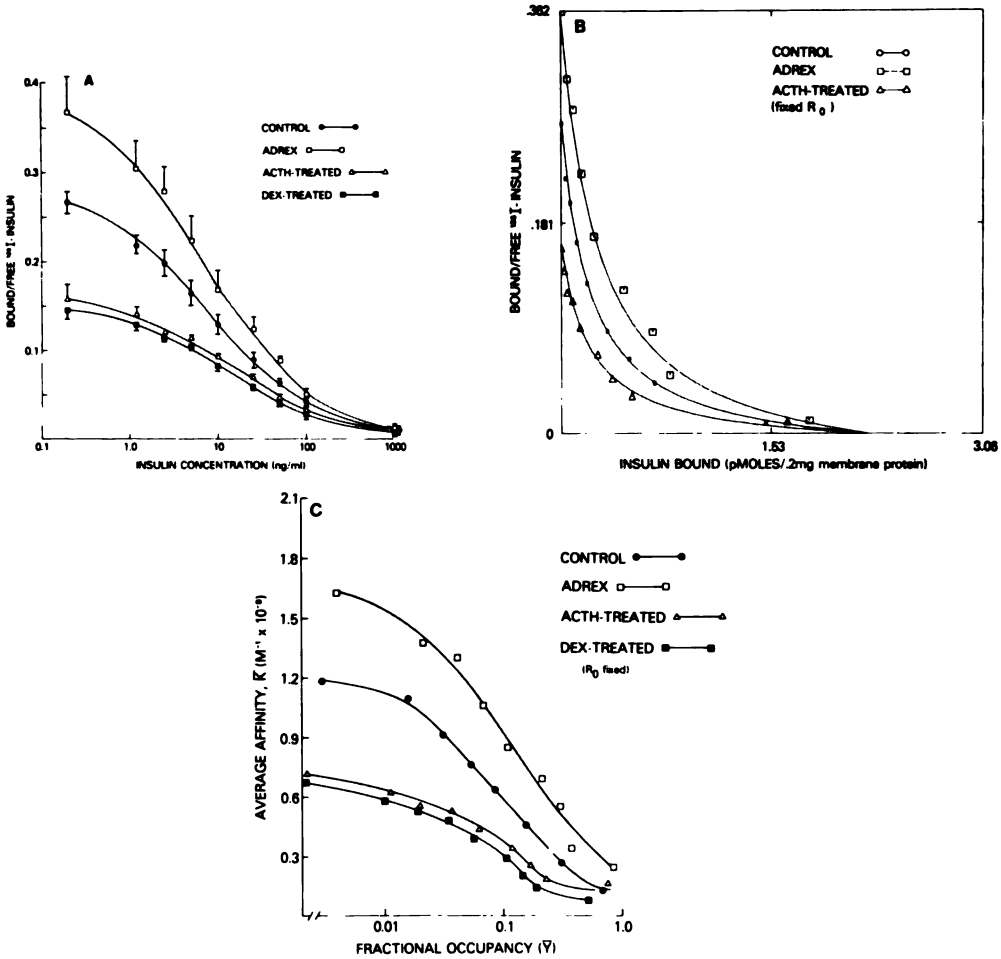


FIG. 4. Effect of steroids on insulin binding. (A) Insulin binding was studied to purified liver plasma membranes of normal rats (control), rats treated with ACTH or dexamethasone (Dex) for 45 days, or adrenalectomized (Adrex) rats. (B) Scatchard plot of the data in (A) fit using a negative cooperative model. (C) Average affinity probe for the data in (B). Adapted from Ref. (15).

TABLE III. DISEASES ASSOCIATED WITH CHANGES IN THE AFFINITY OF THE INSULIN RECEPTOR

Receptor affinity decreased
—Acidosis
—Glucocorticoid excess
—Insulin resistance with anti-receptor antibodies
—Syndrome of insulin resistance and acanthosis nigricans Type B
—Ataxia telangectasia
—Lipoatrophic diabetes (some)
Receptor affinity increased
—Glucocorticoid deficiency
—Isolated GH deficiency (man)

modified by alterations in either receptor number or affinity, and that a variety of factors may regulate the hormone–receptor interaction. An interesting new subset of receptor-related diseases are those in which receptors seem to become the target of a disordered immune response and in which their function is altered by specific antireceptor antibodies. Anti-receptor antibodies have been implicated in the pathogenesis of three diseases: Graves's disease, with antibodies to the thyroid-stimulating hormone receptor; myasthenia gravis, with antibodies to the acetylcholine receptor; and a syndrome of extreme insulin resistance in which antibodies to the insulin receptor have been found (37).

The syndrome of insulin resistance due to anti-receptor antibodies is a rare disorder, but has provided many useful insights into the role of receptors in disease, as well as basic information about the receptor itself. The

hallmark of the syndrome is extreme resistance to both endogenous and exogenous insulin (47). The tissue resistance results in compensatory hyperinsulinemia, with basal and stimulated insulin levels from 10 to 100 times greater than normal. In some patients, the endogenous hyperinsulinemia is sufficient to overcome the insulin resistance, and glucose tolerance may be normal or only mildly impaired. In others, the hyperinsulinemia does not compensate for the insulin resistance, and clinical diabetes results. In these diabetic patients, the resistance to exogenously administered insulin can be extraordinary; one probable case in the literature (48) received up to 177,500 units in one day. None of the known causes of insulin resistance, such as high titers of anti-insulin antibodies, obesity, lipoatrophy, Cushing's disease, or acromegaly, accompany the disorder. In addition, these patients have features suggestive of an immunologic disease, including high erythrocyte sedimentation rate, positive antinuclear and anti-DNA antibodies, leukopenia, alopecia, and arthralgias. Acanthosis nigricans also is present in most patients.

To investigate the possible role of the insulin receptor in this disease, we studied the binding of insulin to its receptor on circulating monocytes in these patients. Like animals with glucocorticoid excess, patients with insulin resistance and anti-receptor antibodies have decreased binding of insulin to its membrane receptors, and this decrease is due to a decrease in receptor affinity (Fig. 5) (47, 49). However, the defect differs from that due to steroids in that only the high-affinity state of the receptor is altered. The receptors behave as if they are "locked" in the low-affinity ($\bar{K}_f$) conformation. All existing data suggest that the underlying receptor is normal. Thus, the defect can be corrected by removing the anti-receptor antibodies by acid wash of the cells or plasma exchange and is absent in cultured fibroblasts from the patients and during remission (49).

The antibodies to the insulin receptor have been detected using two basic assays (Fig. 6). The binding-inhibition assay, initially conceived of by Flier *et al.* (50), is the simplest and may be performed with intact cells, membranes, or even solubilized receptors. The immunoprecipitation assay is theoretically more sensitive, but is considerably more tedious and requires solubilized receptors (51). Thus far most of the antibodies have been IgG in nature, although one patient had IgM antibodies, and in one family with insulin resistance due to anti-receptor antibodies and ataxia telangectasia, the antibodies appeared to be a low molecular weight, monoclonal IgM (52, 53).

Attempts to understand the mechanism by which these anti-receptor antibodies produce an insulin-resistant state were initially confusing. When studied in a variety of systems acutely, the anti-receptor antibodies mimicked insulin action (Table IV) (54–57). This insulin-like effect, however, is transient. Exposure of cells to anti-receptor antibody for 36 hr results in a state of insulin resistance (Fig. 7) (58). This desensitization of the cells occurs without a further change in insulin binding and appears to be an active metabolic process.

Both the acute biological effects and chronic state of desensitization appear to require antibody bivalence (59). Monovalent antibody fragments bind to the cell and block insulin binding and action, but are without biological effect (Fig. 8). Addition of a second

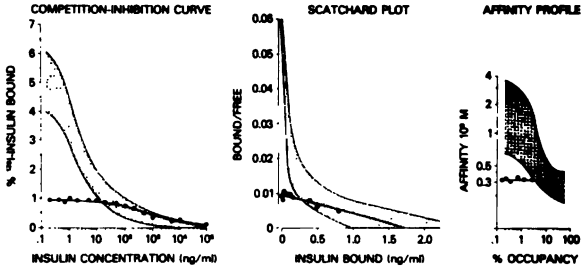
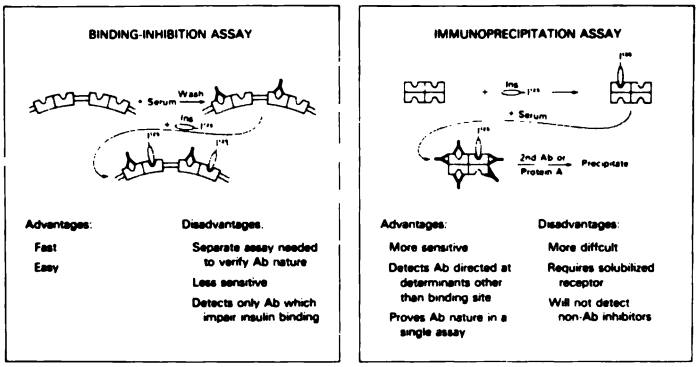


FIG. 5. Insulin binding to circulating monocytes from a patient with anti-receptor antibodies. Insulin binding was studied as previously described. The shaded area is the mean ± SD for 35 normal controls. Note the flat affinity profile which contrasts that seen after glucocorticoids, although in both cases receptor affinity is decreased.



G. 6. Two direct assays for antibodies to the insulin receptor. In the binding-inhibition assay, the cells or branes are first exposed to serum (usually for 60 min at 22°) and washed and then insulin binding is studied. e immunoprecipitation assay, solubilized receptors are prelabeled with ^{125}I-insulin, then serum is added, and inoprecipitation of the complex is effected by addition of a second antibody or protein A–Sepharose.

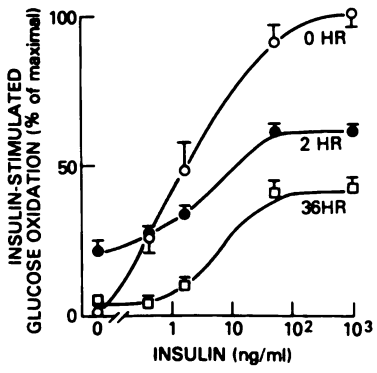
TABLE IV. BIOLOGICAL EFFECTS OF THE ANTI-RECEPTOR ANTIBODIES

ocytes
mulation of 2-deoxyglucose transport, glucose incorporation into lipid and glycogen, and metabolism to CO_2
tivation of glycogen synthase (in the presence and absence of glucose)
mulation of pyruvate dehydrogenase and acetyl-CoA carboxylase
mulation of leucine incorporation into protein
ıibition of lipolysis
ıibition of phosphorylase

L1 Preadipocytes
mulation of 2-deoxyglucose transport and glucose metabolism to CO_2
mulation of lipoprotein lipase

:le
imulation of 2-deoxyglucose transport and glucose incorporation into glycogen
imulation of glycolysis
imulation of glycogen synthase (partial)

r
imulation of AIB transport



IG. 7. Effect of prolonged treatment of 3T3-L1 cells anti-receptor antibody. Cells were pretreated for 0, r 36 hr with anti-receptor antibody, and insulin-ulated glucose oxidation was measured in the usual ion. Adapted from Ref. (58).

antibody which crosslinks the monovalent antibody fragments restores bioactivity.

Summary and perspectives. The insulin receptor is at a crucial step in insulin action serving as the link between extracellular insulin concentration and the metabolic pathways for insulin action. Changes in receptor number and affinity may occur under a variety of influences and play an important role in many disease states. The discovery of antibodies to the receptor has not only proved to be of interest with respect to illucidation of a mechanism for disease, but the antibodies themselves have been a unique probe of receptor structure and function. Recently, using these antibodies we have developed an immunoassay for the solubilized insulin receptor (60). With this assay we should be able to

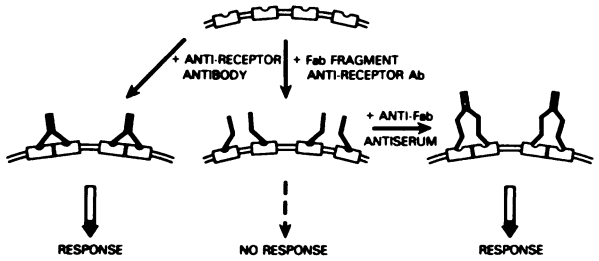


FIG. 8. Proposed mechanism for the biological effects of antibodies to the insulin receptor.

further characterize the receptor, as well as uncover new defects in the receptor which may not be manifest by alterations in insulin binding. The future identification of other factors (including drugs) which regulate receptor concentration or affinity will also be important in our understanding of the hormone–receptor interaction, as well as of the management of disease states.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 22-25 (1979)

Steroid Hormone Receptors and Disease: Breast Cancer (40610)

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Breast cancer is the leading cause of cancer death in women today. Although more than 90,000 new cases are diagnosed and 30,000 women die annually from breast cancer, few major advances have occurred in our understanding of the disease or its treatment until recently. In the past few years, however, basic laboratory research into the factors controlling breast cancer cell growth has been rewarded with significant new information applicable to the clinical care of patients. In fact, as a direct result of laboratory investigation, our approach to the management of this malignancy has changed dramatically. It is the purpose of this review to highlight some of the recent advances on the use of steroid receptors in the management of breast cancer patients and pinpoint the need for future clinical trials.

Cytoplasmic estrogen receptor in primary breast cancer. The most useful criterion to predict the likelihood of recurrence following mastectomy for primary breast cancer is the axillary node status. Patients with tumor-infiltrated axillary nodes are at high risk of returning with metastatic disease (2). This finding has led to the introduction of systemic therapies following mastectomy in node-positive patients to delay or prevent recurrences. Since estrogen receptor (ER) assays have been used with considerable success to predict responsiveness to endocrine therapy in patients with advanced disease (13), it was reasoned that they might be similarly useful in deciding whether or not to include endocrine therapy in an adjuvant regimen. Previous attempts of adjuvant endocrine therapy have been disappointing but probably were inconclusive since stratification of patients according to ER status had not done. As an effort preliminary to an actual adjuvant trial, a series of 145 patients with operable breast cancer and an ER assay of the primary tumor specimen were examined for recurrence and survival (9). The data are unambiguous and are illustrated in Table I. Irrespective of age, nodal status, size of tumor, or location of the tumor in the breast, patients with ER− tumors recurred earlier than ER+ patients. In a recent update of this series (10), Knight and colleagues showed that survival is adversely affected as well in patients with ER− tumors, confirming an earlier report by Walt *et al.* (20). Thus, the ER status of the primary tumor is a useful marker of the biological aggressiveness of the disease.

What then are the implications for adjuvant therapy? We note that in the two most widely publicized trials of adjuvant chemotherapy (1, 3), delay in recurrence is confined to premenopausal women. Since 78% of premenopausal patients receiving combination chemotherapy developed amenorrhea (1), and Rose and colleagues showed that the same drug regimen caused decreased ovarian steroid production and elevated gonadotropin production (17), it is tempting to suggest that the benefits of cytotoxic adjuvant therapy in premenopausal patients are due to drug-induced ovarian failure. However, a recent preliminary report (19) indicates that chemotherapy-induced ovarian failure occurs only in premenopausal patients over the age of 40 and could not explain the delay in recurrence observed in younger patients receiving chemotherapy.

An alternative line of reasoning might be considered to explain the chemotherapy benefits in premenopausal women. First, compared to older patients, premenopausal patients are more likely to have ER− tumors; those with ER+ tumors have considerably lower absolute ER values, implying a greater precentage of ER− cells within the tumor. Next, ER− tumors have a higher thymidine labeling index than ER+ tumors, indicating that more cells within the tumor are undergoing DNA synthesis (16). This would support the clinical observations referred to above that ER− tumors are more aggressive in their

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behavior. Since in many tumor sys- has been shown that tumors with the ıpid doubling times are the most sen- › chemotherapy, the idea emerges that umors should be most responsive to herapy.

ıen what are the questions to be an- regarding receptor status and adju- erapy? As shown in Table II we would / divide node-positive patients into nd ER+ groups instead of the usual :ation of pre- and postmenopausal. ıakes the assumption that tumor ag- :ness and response to both endocrine emotherapy are determined primarily status rather than menopausal status. presents a departure from traditional g, but in view of the arguments listed it may have considerable merit. ER− s will not respond to endocrine therapy : data of Meyer *et al.* (16) indicate that ggressive ER− tumors have high thy-

LE I. ER AND THE PROGNOSIS FOR EARLY RECURRENCE OF BREAST CANCER[a]

	Percentage recurrence at 18 months	
Category	ER−	ER+
<50	34	14
>50	35	8
es 0	12	6.5
1–3	38	12.5
≥4	62	27.0
<2 cm	33	0
>2 cm	31	14

pted from Ref. (9).

: II. A SCHEME FOR AN ADJUVANT THERAPY N AXILLARY NODE-POSITIVE BREAST CANCER PATIENTS BASED ON ER STATUS

Menopausal status	Plan
Premenopausal	Chemotherapy A vs chemotherapy B
Postmenopausal	Chemotherapy A vs placebo
Premenopausal	Ovariectomy + (antiestrogen or medical adrenalectomy) vs chemotherapy A
Postmenopausal	Antiestrogen or medical adrenalectomy vs placebo

midine labeling indices which should respond to chemotherapy. Since no benefit has yet been shown in postmenopausal patients from adjuvant therapy, a placebo group is justified.

In ER+ patients we consider menopausal status because of the important ovarian source of estrogens and the results of current adjuvant trials showing a benefit from chemotherapy only in the premenopausal group (1, 3). Premenopausal patients who have ER+ tumors would be randomized between an endocrine therapy and combination chemotherapy. The logical choice for endocrine therapy would be ovariectomy to remove ovarian sources of estrogens. However, it is well known that when patients with advanced disease eventually relapse following ovariectomy, another objective remission can be obtained by removing the adrenal glands. The rationale is that the adrenals secrete appreciable quantities of androstenedione which is peripherally converted to estrogen, resulting in tumor growth. So in addition to ovariectomy we might use another tactic to decrease adrenal androstenedione production or block its effect at the tumor cell. Either approach is feasible and has been demonstrated to be effective. The combination of aminoglutethimide and hydrocortisone can effectively block adrenal estrogen precursor production and induce tumor regression in postmenopausal women (18). Alternatively, the estrogen derived from the adrenal can be prevented from stimulating tumor cell growth by antiestrogens, a relatively new class of compounds which exert their effects at the ER level in tumor cells (5). In view of the above, a combination of ovariectomy plus either antiestrogen or aminoglutethimide and hydrocortisone versus combination chemotherapy seems warranted in the premenopausal ER+ patient.

For postmenopausal ER+ patients, the ovarian source of estrogen is virtually nonexistent. Either antiestrogens or aminoglutethimide and hydrocortisone would be effective in countering any adrenal estrogen precursor production or action. Since chemotherapy has not been shown to be of benefit in this group, a placebo control arm is justified.

It should be emphasized that this scheme for future adjuvant trials is tentative at pres-

ent. There are currently many adjuvant trials underway which hopefully will provide new information to refine our thinking. On the other hand, most current trials have not taken ER stratification into account which may blunt the interpretation of the results.

Cytoplasmic receptor in advanced breast cancer. The measurement and use of cytoplasmic ER in deciding treatment strategy for patients wiith advanced breast cancer requires little discussion. It has taken much of the empiricism out of the decision-making process of whether to use or reject endocrine therapy in a particular patient. What is relatively new is that the response rate to endocrine therapy is proportionate to the actual quantitative amount of ER in the tumor specimen (4, 15). This is illustrated in Table III. Patients with very low amounts of ER respond infrequently whereas patients with very high levels of ER have very high response rates and intermediate ER values yield intermediate response rates. What therapy should we recommend based on quantitative ER values? In the case of low values the choice of chemotherapy is obvious since endocrine therapy will not be effective. In the intermediate group, the response rate to endocrine therapy is not sufficiently high to be dogmatic. Many of these patients will have good objective remissions to endocrine therapy. It would seem that a trial of endocrine therapy, preferably not major ablative surgery, is indicated with the plan to initiate chemotherapy if progression is observed. Alternatively, the simultaneous use of endocrine therapy and chemotherapy might be very effective. In the group with very high ER values, endocrine therapy alone is probably indicated in view of the very high response rates, reserving chemotherapy for subsequent progression. It is with this latter group that major ablative endocrine surgery could be considered since the associated morbidity may be justified.

Progesterone receptors and response to therapy in advanced breast cancer. In view of the success of ER to help select or reject endocrine therapy in patients with advanced breast cancer, many investigators have reasoned that the measurement of other receptors might even strengthen the correlations with endocrine therapy. We chose progesterone receptor (PgR) since in normal reproductive tissue the synthesis of PgR is dependent on estrogen stimulation (8). We were able to show that PgR could be measured in human breast tumor cytosols (17) and that PgR synthesis is stimulated by the direct addition of estradiol to human breast cancer cells in culture (6).

TABLE III. ER Level in Metastatic Biopsy and Response to Endocrine Therapy

ER value[a]	Response
<3	2/33 = 6%
3-10	24/52 = 46%
11-1000	21/26 = 81%

[a] fmole/mg cytosol protein; adapted from Ref. (15).

Numerous laboratories are now accumulating data on the correlation of ER and PgR and the response to endocrine therapies. A listing of all published data and personal communications is shown in Table IV. Since patient selection, assay methods, and criteria for objective response are probably different in each series, it is not legitimate to pool the data as in Table IV to arrive at definite conclusions. On the other hand, it is useful to illustrate the early results from a variety of centers. Several points are worth noting. First, the response rate of 14% in ER−, PgR- patients is high if one considers the response rates reported for ER alone in the past, but is best explained by the unusual results from one group in the series. Second, the presence of both receptors in a tumor specimen is associated with the highest response rates. Since we have previously shown that PgR is more likely to be present in tumors with high ER content (14), and that high ER content predicts for a higher response rate, it is not clear at this point whether PgR is just telling us that the ER content of the tumor is high and that a good response is to be expected, or rather that PgR is providing additional information independent of ER. This must be dealt with in future analyses. Now that ER and PgR data are beginning to appear from a variety of independent sources, we should in the very near future be able to conclude whether the measurement of PgR in addition to ER is of value.

Summary. The use of cytoplasmic estrogen receptor (ER) to help select patients with advanced breast cancer for endocrine therapy is well established and whenever possible should be part of the routine evaluation.

.E IV. OBJECTIVE RESPONSE TO ENDOCRINE THERAPY (231 CASES) BY RECEPTOR CONTENT

	ER−, PgR−	ER−, PgR+	ER+, PgR−	ER+, PgR+
et al.	0/11	—	7/17	13/16
in *et al.*[a]	0/7	1/1	1/9	16/18
.[b]	1/5	1/2	1/7	8/9
al.[c]	5/17	—	0/3	4/7
et al. (11)	—	0/1	1/3	1/3
o *et al.* (12)	2/20	0/1	9/25	12/20
al. (21)	1/3	1/1	1/7	13/18
	9/63 = 14%	3/6	20/71 = 28%	67/91 = 74%

ommunication.
I. B., Redgrave, S., Rubens, R. D., Millis, R., and Hayward, J. A., manuscript in preparation.
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ntial uses of steroid receptors are The data suggest that an ER deter- n the primary tumor may be very in designing new adjuvant trials. : presence of progesterone receptor signals the presence of a higher t and very likely a favorable re- ndocrine therapy.

the author's own laboratory were sup- : by the NIH (CB 23862, CA 11378) and i Cancer Society (BC-23G). I wish to thank ein, King, Skinner, and Matsumoto for with their data prior to publication, and naud of Roussel Uclaf for providing the progesterone receptor studies.

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Acetylcholine Receptors and Myasthenia Gravis (40611)

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Myasthenia gravis is a neuromuscular disorder characterized by weakness and fatigability of muscles. The clinical features of myasthenia were first noted by Willis (27) in 1672 and were thoroughly described by the turn of the century (4). The neuromuscular junction was implicated in the disease process more than 40 years ago, but it was not until 1973 that the acetylcholine (ACh) receptors were identified as the precise site of the defect (13). Important advances have been made in understanding the mechanism of the autoimmune attack on ACh receptors (7), but many important questions remain.

In order to understand the pathogenesis of myasthenia gravis, it is necessary to review briefly the physiology of transmission of ACh at the neuromuscular junction (14) (Fig. 1). ACh is synthesized at motor nerve endings and is packaged in membrane-bounded vesicles, or "quanta," containing approximately 10,000 molecules each. ACh is released spontaneously or in response to electrical impulses conducted by the motor nerves. The released ACh crosses a narrow space and combines with ACh receptors which are clustered at the peaks of postsynaptic folds immediately opposite the ACh release sites. The combination of ACh with receptors causes a rapid increase in permeability of the membrane to sodium and potassium ions, resulting in electrical depolarization. The spontaneous release of individual quanta of ACh gives rise to depolarizations of the order of 1 mV called miniature end-plate potentials, or "mepps." Nerve impulses release approximately 150–200 quanta of ACh, giving rise to much larger end-plate potentials (epps), which trigger muscle action potentials and, consequently, muscle contractions.

In the early 1960s Elmqvist and colleagues observed that mepp amplitudes in myasthenic patients' muscles was reduced to about 20% of the normal value (12). They postulated that this was due to a presynaptic defect in the motor nerve, with a decreased of ACh contained in each quantun ever, we wondered whether the lo amplitudes might be accounted fo duced numbers of available ACh re We tested this hypothesis with the a bungarotoxin (α-BuTx) a purified fra a snake venom that binds specifical versibly, and quantitatively to ACh r at neuromuscular junctions. α-BuTx ily labeled with ^{125}I and can therefore to quantify ACh receptors.

We obtained "motor point biopsie taining neuromuscular junctions, fro tients with myasthenia gravis and fr mal and disease controls. The musc saturated with ^{125}I-α-BuTx *in vitro*, excess was removed by washing. The of ACh receptors per neuromuscular j was calculated from the bound radic and the number of neuromuscular j present. In the normal muscles, the number of receptor sites per neuron junction was 3.7×10^7, while the my junctions had only 0.7×10^7, less th of the normal number (11, 13). This suggested a defect of ACh receptors.

In order to determine whether a re of available ACh receptors could acc the physiological and clinical abnor in myasthenia, we produced an expe model in rats by blocking a proportio muscle ACh receptors with anothe specific snake toxin, α-cobra toxin (2 rats were weak, requiring artificial tion. They showed the typical physi features of myasthenia, including de tal responses on repetitive nerve stim curare sensitivity, and improvement ticholinesterase agents, which are us tinely as clinical diagnostic tests fo thenia in man. These findings confir theory that a reduction of ACh r could account fully for the physiolog tures of human myasthenia gravis.

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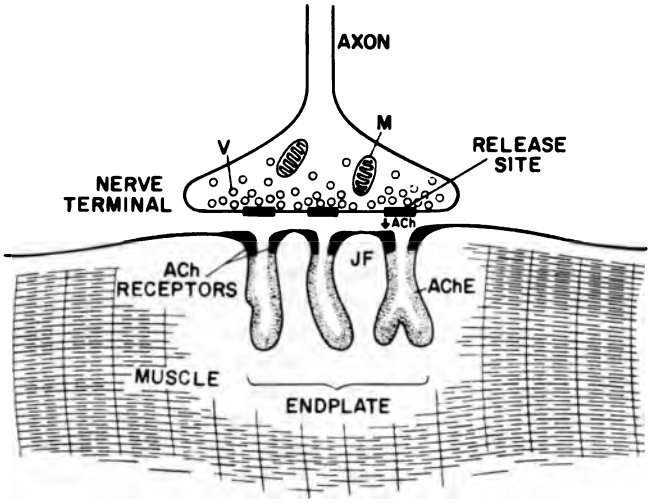

1. Diagram of neuromuscular junction. Vesicles (V) release their acetylcholine (ACh) contents at specialized ıites. After crossing the narrow synaptic space (path indicated by arrow) ACh reaches the ACh receptors, re most densely situated at the peaks of the junctional folds (JF). Acetylcholinesterase (AChE) in the clefts hydrolyzes the ACh. M, Mitochondria. Reprinted with permission from the N. Engl. J. Med. 298, 136 (1978).

v is the ACh receptor defect produced n? Several clues suggested an autoim- process. The association between myas- gravis and other autoimmune disor- 23), as well as the high proportion of : abnormalities in patients with myas- (5), first suggested an autoimmune er. A further important clue that the ımune attack might be directed against eceptors came from the work of Patrick indstrom who were attempting to raise dies to purified ACh receptors (derived he electric organs of electric eels, one richest sources of ACh receptors). They that rabbits injected with ACh receptor und's adjuvant developed a condition ;ous to myasthenia gravis (20). Further, dies against ACh receptors were iden- n the serum of myasthenic patients (1). question of whether the circulating dies are instrumental in causing the ɔr deficit, or merely represent a second- sponse to receptor damage caused by ɔther agent, is of great importance in tanding the pathogenesis of myas- . One well-known clinical observation ted that circulating antibodies might hogenic: Some infants born to myas- mothers are affected by myasthenic ess during the neonatal period, with te recovery after several weeks (18). However, numerous experimental attempts to transfer a myasthenia-producing factor from humans to animal preparations in the past had failed (19). In most studies of attempted transfer, animals or nerve–muscle preparations were exposed to the myasthenic serum for a brief time, usually minutes or a few hours. Based on the idea that a relatively prolonged period of exposure might be required for the effect to take place, we undertook to transfer immunoglobulins from the serum of human myasthenic patients to experimental mice (26). Daily injections of immunoglobulin (Ig) from myasthenic patients or controls were given to mice in doses sufficient to maintain levels of IgG equivalent to those in the humans, for 1 to 14 days. Some of the experimental mice became clinically weak and showed decremental responses on repetitive nerve stimulation. Passive transfer of Ig from 94% of the patients produced a reduction of ACh receptors, a reduction of mepp amplitudes, or both in the muscles of the recipient mice. Serum from only 1 of 16 patients failed to produce a reduction of either ACh receptors or mepp amplitudes. Injections of Ig from control individuals had no effect on the recipient mice (25).

By the use of this passive transfer model, we have found that only the IgG fraction produced the features of myasthenia, while

other serum immunoglobulins had no effect. Furthermore, the early part of the complement system enhanced the effect of IgG. Depletion of C3 (in the recipient mice) diminished, but did not eliminate, the effect of myasthenic patients' IgG on ACh receptors. However elimination of the latter part of the complement system (C5) did not reduce the effect of myasthenic patients' IgG. Thus, IgG appeared to play an important role in the pathogenesis of myasthenia gravis, enhanced by the early part of the complement cascade.

Theoretically, circulating antireceptor antibody might reduce the number of available ACh receptors by several different mechanisms:

(a) It might *block* the active sites of the receptors by binding at or near them.

(b) It might *alter the turnover* of ACh receptors, either by increasing the rate of degradation, or by decreasing the rate of synthesis, or both.

Evidence from tissue culture studies now suggests that both accelerated degradation of ACh receptors (2, 3, 10, 15) and receptor blockade (10, 15) may be involved. The methods used for measuring receptor degradation in tissue culture have previously been well worked out. Skeletal muscle from rat fetuses is grown in culture, and the ACh receptors are labeled with ^{125}I-α-BuTx. As the labeled receptors undergo degradation, the attached ^{125}I-α-BuTx is broken down to iodotyrosine, which is released into the culture medium. The rate of receptor degradation can be determined from the amount of radioactivity released into the medium per unit time; it is normally approximately 4% per hour for rat skeletal muscle. When Ig from myasthenic patients was added to the cultures, receptor degradation rate increased two- to threefold, as compared with cultures treated with Ig from control patients. The antibody-induced acceleration is triggered by IgG alone, without requiring other humoral or cellular components of the immune system. The antibody alters the receptors so that they are preferentially degraded at the rapid rate. Only those receptors with bound antibody are rapidly degraded, while other receptors in the same cultures are degraded at normal rates (8). This indicates that the effect is not due to an overall acceleration of the cells' ACh receptor-degrading mechanisms, but rather to a specific mechanism involving ACh receptor-antibody complexes.

We wondered whether this effect might be due to the ability of antibodies to crosslink antigens (in this case ACh receptors). Accordingly, we prepared various divalent (Fab'$_2$) and monovalent (Fab) fragments from myasthenic IgG (9). The divalent fragments produced acceleration of receptor degradation, while the monovalent fragments did not (Fig. 2). The fact that crosslinking alone accounted for the effect was demonstrated in two ways:

(i) In one series of experiments monovalent antibodies were first added to the cultures. The cultures were then incubated with a second, "piggyback" antibody directed against the antibody fragments (not against

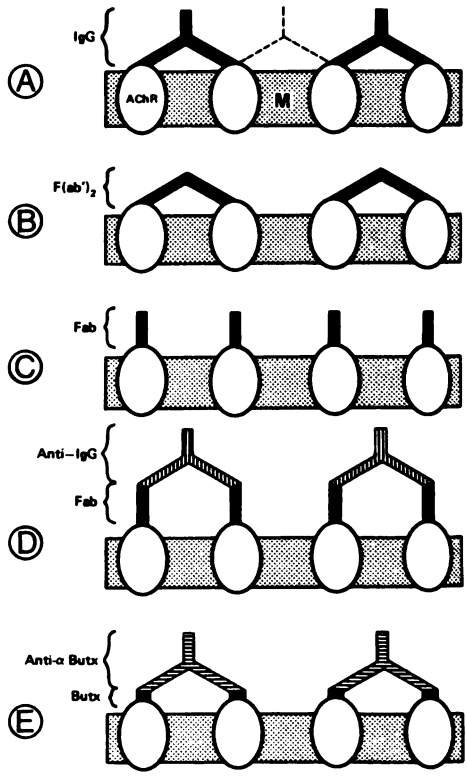

FIG. 2. Diagrammatic representation of crosslinking of receptors by the various antibody fragments. M, Muscle membrane; AChR, acetylcholine receptor; α-BuTx, α-bungarotoxin. Acceleration of degradation was observed in the experimental conditions shown in A, B, D, and E. Fab alone failed to accelerate acetylcholine-receptor degradation. Reprinted with permission from the N. Engl. J. Med. 298, 1120 (1978).

receptors). The effect of the second ıdy was to crosslink the monovalent ents, which in turn were bound to the ors. This indirectly crosslinked the ACh ors and had the same accelerating effect Ch receptor degradation as the original nt antibodies.

We prepared antibodies against α- by immunizing rabbits with α-BuTx in d's adjuvant. When cultures labeled ^{125}I-BuTx were treated with the anti- antibodies, the degradation rate was accelerated two- to threefold. In this ıe antibodies were bound to the α-BuTx ıus indirectly produced crosslinking of receptors. Taken together, these findtrongly suggest that the ability of anti- ; from myasthenic patients to crosslink receptors accounts for their effects on or degradation. It remains to be shown rosslinking induces accelerated receptor dation. It is possible that the crosslinked gates may be recognized by the muscle ırane, because of altered size or mobil- ıd selected for preferential degradation. ıatively, the crosslinked receptors may graded by the normal process, but the er of receptors ingested in each "bite" l be increased because they are bound ıer. We have not yet measured the numf ACh receptors crosslinked together, ıis model would predict aggregates of o four, based on the 2.5- to 3.5-fold ıse of degradation rates observed.

he culture system, the accelerated degon process accounts for part of the loss h receptor sites observed after exposure asthenic immunoglobulin. In addition, ade of receptor sites by the antibody has shown to occur in skeletal muscle cul- as well as in other assay systems (1, 10, here is evidence that the blocking effect to attachment of the antibody near, than directly at, the active site of the or, producing steric hindrance of the site (17).

ıthesis of ACh receptors does not appear affected by immunoglobulin from henic patients. Using the indirect methow available, we have found no change ıthesis and incorporation of new recepn the rat muscle culture system after ıent with immunoglobulin from myasthenic patients (8).

These effects of myasthenic immunoglobulin on receptor degradation and blockade apply not only to the culture system in which they were first tested, but also to ACh receptors at intact neuromuscular junctions. Although there are important differences between *extrajuntional* receptors (like those found in cultured myotubes) and ACh receptors at innervated neuromuscular junctions, the same effect of accelerated receptor degradation occurs *in vivo* at intact neuromuscular junctions (24).

Although more is known about the ACh receptor and the humoral autoimmune mechanisms in myasthenia gravis than in any other receptor disorder, many important questions remain. For example, it is not yet known how the autoimmune process originates. The juxtaposition of muscle-like cells that bear ACh receptors (16) and immunocompetent cells in the thymus suggests that the thymus may be the arena in which autoimmunity originates in this disease. The possibility that an autoimmune process may be triggered by an alteration in either the "myoid cells" or the thymic lymphocytes is plausible (7), and it has been suggested that a viral infection may trigger the autoimmune response (6). Another unsettled question is the range of different immune mechanisms that may operate in patients with myasthenia gravis. Although the ACh receptor is probably always the target of the autoimmune process in myasthenia, it is likely that the specific immune mechanisms used differ in individual cases. The role of cell-mediated mechanisms in myasthenia has not yet been well studied. It is known that lymphocytes from myasthenic patients undergo blast transformation in the presence of purified ACh receptors (21). However, it is not yet known whether the sensitized lymphocytes are T cells or B cells, and whether they are capable of participating in cell-mediated effector mechanisms (7).

Conclusions. This review has dealt with recent advances in the understanding of myasthenia gravis. The fundamental abnormality affecting the neuromuscular junctions of myasthenic patients is a reduction of ACh receptors, due to an autoimmune attack directed against the receptors. Antibodies to ACh receptors are present in most patients,

and there is evidence that they have a pathogenetic role in the disease. The mechanism of antibody action involves acceleration of the rate of degradation of ACh receptors, attributable to crosslinking of the receptors by the antibody. In addition, antibodies produce blocking of the ACh receptors. The possibility that cell-mediated mechanisms may participate in the immune responses in some myasthenic patients remains to be explored. Perhaps the most important gap in the present understanding of myasthenia gravis concerns the origin of the autoimmune process, although there is in direct evidence that the thymus gland may be involved. Future research will be directed toward developing therapeutic methods to deal with the specific immunological abnormalities of individual myasthenic patients.

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S OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 31-33 (1979)

s of Nifedipine on Prolactin, Growth Hormone, and Luteinizing Hormone Release by Rat Anterior Pituitary *in Vitro* (40612)

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well known that Ca^{2+} plays an impor- e in hormone release from the anterior y (1–8); however, the mechanism of ction on the anterior pituitary cells s to be clarified. Douglas *et al.* (9, 10) ed that hormone release from the neu- physis and adrenal medulla is initiated olarization of the cell plasma mem- Depolarization induces a permeability in the plasma membrane resulting in ux of Ca^{2+} into the cells, and this in- ay then initiate the intracellular hor- eleasing mechanism.

ie present study, we investigated the of nifedipine, a Ca^{2+} blocker, on *in* lease of prolactin, GH, and LH from erior pituitary.

rials and methods. Male Sprague– rats, weighing 180–200 g, were used. imals were decapitated, and the ante- uitary was immediately removed and d to equal halves. The two halves of tuitary were placed in a beaker with f Krebs–Ringer bicarbonate medium ing 0.25% bovine serum albumin G–BSA). After two 1-hr preincubation in a metabolic incubator at 37° under and 5% CO_2 gas, the medium was d with fresh KRBG–BSA (1 ml) and ed for 1 hr (first incubation). The ion medium was collected. Then 1 ml BG–BSA containing the test material ded to the beaker and incubated for r 1 hr (second incubation), and the ion medium was collected. The t of pituitary hormone released during ond 1-hr incubation was compared at released during the first 1-hr incu- period and expressed as a percentage released in the first hour.

lipine (Adalat, Bayer Pharmacol. Co.) solved in a vehicle of 15% polyethyl- ene glycol 400, 15% ethanol, and 70% distilled water in concentrations of 100, 10, and 1 μg/ml: Each solution was diluted 10 fold with KRBG–BSA, and then 0.1 ml of solution containing nifedipine was added to 0.9 ml KRBG–BSA medium. The vehicle was also diluted with KRBG–BSA 10-fold, and 0.1 ml of the diluted vehicle was added to samples without nifedipine. Prolactin, GH, and LH levels were determined in each incubation medium by specific radioimmunoassays using rat prolactin, GH, and LH radioimmunoassay kits supplied by NIAMDD. Duncan's new multiple range test was used for comparing the mean release of prolactin, GH, or LH in each beaker.

Results. Nifedipine on prolactin release in vitro. Nifedipine at 10, 100, and 1000 ng/ml progressively inhibited prolactin release in a dose-related manner (Table I). TRH at 100 ng/ml appeared to increase prolactin release, but this effect was not statistically significant compared to the control. The slight stimulation of prolactin release induced by TRH was also inhibited by nifedipine.

Nifedipine on GH release in vitro. Nifedipine at 10, 100, or 1000 ng/ml had no effect on GH release. TRH at 100 ng/ml significantly increased GH release compared to the control. Nifedipine at 1 μg/ml blocked the TRH-induced GH release.

Nifedipine on LH release in vitro. Neither nifedipine nor TRH affected LH release from the anterior pituitary. LH-RH at 100 ng/ml caused a fivefold increase in LH release. Nifedipine at 1 μg/ml significantly blocked the release of LH induced by LH-RH.

Discussion. Nifedipine at 10, 100, or 1000 ng/ml inhibited the release of prolactin, but not that of GH or LH, from rat anterior pituitary tissue. Nifedipine at 100 or 1000 ng/ml blocked not only the TRH-induced GH

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TABLE I. EFFECTS OF NIFEDIPINE ON PROLACTIN, GH AND LH RELEASE IN VITRO RAT PITUITARY TI

Treatment	Dose (ng/ml)	No. beakers	(2nd incubation/1st incubation) × 100		
			Prolactin	GH	LH
(1) Control	—	4	97.0 ± 6.8[α]	102.6 ± 5.0	114.8 ± 8.6
(2) Nifedipine	10	4	81.9 ± 16.4	101.9 ± 12.0	106.9 ± 13.9
(3) Nifedipine	100	4	52.2 + 4.1**	86.4 ± 12.4	122.3 ± 7.5
(4) Nifedipine	1000	4	41.4 ± 5.7**	104.1 ± 4.3	118.3 ± 16.8
(5) TRH	100	4	124.3 ± 19.6	144.1 ± 4.8*	109.6 ± 8.8
(6) Nifedipine TRH	100 100	4	88.5 ± 5.6	133.7 ± 6.3	113.2 ± 4.8
(7) Nifedipine TRH	1000 100	4	67.2 ± 1.0[α]	104.5 ± 8.1[β]	108.4 ± 8.9
(8) LH-RH	100	4	85.2 ± 7.8	117.7 ± 13.1	562.7 ± 61.8
(9) Nifedipine LH-RH	1000 100	4	41.7 ± 3.8	102.8 ± 4.8	398.8 ± 44.3

[α] Mean ± SEM.
* $p < 0.05$ vs control.
** $p < 0.01$ vs control.
[α] $p < 0.01$ vs (5).
[β] $p < 0.05$ vs (5).
[γ] $p < 0.05$ vs (8).

but the LH-RH-induced LH release as well. Nifedipine at 1000 ng/ml also inhibited the slight stimulation of prolactin release induced by TRH. Nifedipine has been shown to have a strong blocking effect on excitation–contraction coupling in myocardium without inhibiting the electrical excitation process (11). It inhibits excitation–contraction coupling by blocking Ca^{2+} influx from the extracellular space.

It is well known that Ca^{2+} is involved in the releasing mechanism of pituitary hormones. Removal of Ca^{2+} from the incubation medium inhibits their release. Samli and Geshwind (2) reported the stimulatory effect of high K^+ medium on LH secretion and demonstrated the Ca^{2+} requirement for LH-releasing action in response to both high K^+ medium and hypothalamic extracts. Wakabayashi *et al.* (3) found that Ca^{2+} uptake initiates the release of gonadotropins. Vale *et al.* (1) also reported that Ca^{2+} is required for TRH-induced secretion.

An interesting finding was that nifedipine at 10 to 1000 ng/ml did not affect the release of GH or LH, but that prolactin release was blocked by nifedipine in a dose-related manner. MacLeod and Fontham (5) found that the absence of Ca^{2+} from Krebs–Ringer bicarbonate medium greatly diminished prolactin synthesis and release. GH synthesis and release were also decreased by incubation in Ca^{2+}-free medium, but the relative decrease was greater with prolactin than with They also found that a fivefold higher concentration increased the synthesis a lease of prolactin without affecting th thesis of GH. Our results and the d Macleod and Fontham indicate tha mechanism of prolactin release from t terior pituitary may be more closely in with Ca^{2+} influx from extracellular flui in stimulus–secretion coupling than t lease of GH or LH.

Eto *et al.* (12) reported that verapa Ca^{2+} blocker, did not inhibit the basal r of ACTH, GH, or TSH. They also re that verapamil had no effect on ACT lease induced by crude hypothalamic e or synthetic lysine vasopressin, and tha apamil did not inhibit the TSH relea duced by synthetic TRH or the GH r induced by crude hypothalamic extract thus concluded that hypothalamic rel factors did not affect the process of stin secretion coupling by promoting a s calcium influx. Zimmerman and Fleis reported that the ACTH release indu vasopressin decreased after incubati Ca^{2+}-free medium.

Wakabayashi *et al.* (3) also reporte the effects of releasing factors on the L FSH response were only partially inl in Ca^{2+}-free medium, while the action K^+ was completely abolished. In this nifedipine at relative large doses com

ıhibited the release of TRH-induced GH nd partially, but significantly, prevented the .H-RH-induced LH release, and also nife-ipine at a large dose inhibited the release of ıe TRH-induced prolactin release.

Summary. The release of prolactin, GH, nd LH was monitored by radioimmunoas-ay in rat anterior pituitary tissue incubated ı Krebs–Ringer bicarbonate medium. Nife-ipine, a strong Ca^{2+} blocker, at 10, 100, and 000 ng/ml inhibited the release of prolactin n a dose-related manner, but did not inhibit he release of GH and LH. This drug also)locked the TRH-induced GH release and he LH-RH-induced LH release. These re-ıults indicate that the release of prolactin may)e more closely tied to Ca^{2+} influx from :xtracellular fluid and with stimulus–secre-ion coupling than the release of GH and LH ınd that Ca^{2+} may also play a role in the elease of anterior pituitary hormones in-luced by releasing hormones.

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Metabolic and Structural Requirements for Concanavalin A Capping in Phagocyti (40613)[1]

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The distribution of concanavalin A (Con A) receptors on the surface membrane of polymorphonuclear leukocytes is dependent upon the state of microtubule and microfilament assembly (1). Binding of fluorescein isothiocyanate–Con A (FITC–Con A) to its surface receptors induces polymerization of cytoplasmic tubulin into microtubules in polymorphonuclear leukocytes. In turn, lectin-treated polymorphonuclear leukocytes display a uniform distribution of FITC–Con A (2). Colchicine inhibits tubulin polymerization and stimulates lateral movement of Con A receptors into surface caps (2). Capping can be prevented in polymorphonuclear leukocytes by exposing the cells to cytochalasin B, a fungal metabolite known to fragment actin-microfilaments (3). In contrast to polymorphonuclear leukocytes, however, FITC–Con A capping is promoted by cytochalasin B even in the absence of colchicine in alveolar macrophages (4). The difference in capping response to cytochalasin B between polymorphonuclear leukocytes and alveolar macrophages may reflect dissimilarities in the major metabolic pathways employed by each cell to obtain energy, distinctive isoactin forms between each cell, or varied actin-microfilament topographical relationships in the cytoskeleton of the cell. In this study, we report the effect of cytochalasin B and metabolic inhibits on FITC-Con A capping guinea pig polymorphonucl kocytes, alveolar macrophages, and neal monocytes. We also identify an pare isoactin forms from each cell ty

Materials and Methods. Polymor clear leukocytes and peritoneal mo were obtained from male albino guin 18–72 hr after intraperitoneal injectic and 1.2% sodium caseinate, respectiv Alveolar macrophages were harves bronchopulmonary lavage of guinea receiving caseinate (6). Cells were three times and suspended in iced Ringer phosphate buffer, pH 7.4. Cel was 95% as determined by Wrights an ocyte-specific α-naphthyl esterase sta Cell viability was 98–100% as determ exclusion of 0.1% trypan blue. The c of lectin receptors to move into polar the cell membrane was determined serving the distribution of FITC–Co previously described (4). Cells wer bated 15 min at 37° with or without drugs, stained 5 min with 50 μg FIT A, and fixed in 2% formaldehyde. Af leting, the percentage of capped ce determined. The energy requirement capping response to colchicine was mined by preincubating cells for 45 m either 1 m*M* potassium cyanide or 5 deoxyglucose prior to addition of col For determination of isoactin forms, cell homogenates of 1×10^7 cells we pared by homogenation in a Dounce h enizer (8). To isolate the cellular ac homogenates were clarified by centrif at 1000*g* and the supernatant was m m*M* in potassium chloride and sti room temperature for 1.5 hr. The s was centrifuged at 80,000*g* for 3 hr (4 the resulting pellet was dissolved in tissue grinder with a small volume of

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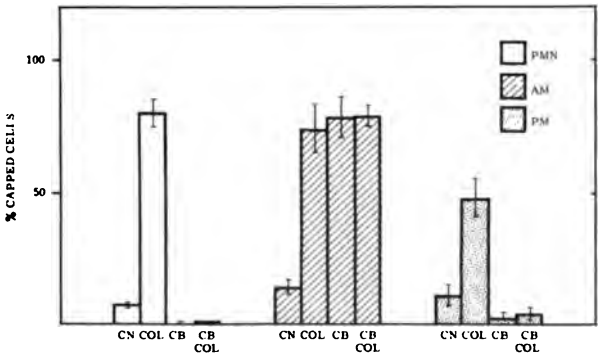
ntaining 0.2 m*M* adenosine triphos-
10 m*M* dithiothreitol, 0.2 m*M* calcium
e, 2 m*M* Tris–HCl, pH 7.0, and was
d against that buffer for 3 days. The
te was centrifuged at 80,000*g* for 3 hr
e supernatant was applied to DNase
/ column (9). The peak fractions from
finity column were pooled, desalted,
alyzed against distilled water for 18 hr.
mples were lyophilized and redissolved
sodium dodecyl sulfate which was dis-
with excess Triton X-100 (10). Isoelec-
using in polyacrylamide gels contain-
ea and Triton X-100 was performed
ing to the method of O'Farrell (11),
that the gel lengths were increased to
and the focusing increased to 9000–
V-hr in order to obtain better resolu-
the actin forms.

ılts. Figure 1 shows the response of
ell type to various drugs. Treatment of
ith colchicine, a drug known to inhibit
sembly of microtubules, induced the
ion of Con A caps in all cell types
However, the response of peritoneal
ytes to cytochalasin B as well as to
alasin B and colchicine was similar to
the polymorphonuclear leukocytes. As
ısly reported, cytochalasin B induced
capping in alveolar macrophages,
as seen in Fig. 1, cytochalasin B pre-
vented colchicine-induced Con A capping in both the polymorphonuclear leukocytes and peritoneal monocytes. In an effort to implicate differing isoactin forms in the observed disparity of the capping response to cytochalasin B, we isoelectrophoresed homogenates from each cell type. As noted in Fig. 2, the isoelectric focusing patterns of peritoneal monocytes and alveolar macrophages were similar. In particular, two bands corresponding to β- and γ-actin were present in both cell types. Similarly, while the isoelectric focusing pattern of the polymorphonuclear leukocyte homogenate was clearly distinct from those of alveolar macrophages and peritoneal monocytes, these cells also contained both cytoplasmic actins (β and γ). As observed in other nonmuscle cells, β-actin comprises the majority of the total actin and none of the skeletal muscle actin form (α) was present in any of the three cell types.

The difference in energy metabolism among the three phagocytic cell types suggested a possible link between the varied response to cytochalasin B and the major metabolic source of energy in each cell type. Indeed, as seen in Fig. 3, the energy required for colchicine-induced capping differed among polymorphonuclear leukocytes, peritoneal monocytes, and alveolar macrophages. In both polymorphonuclear leukocytes and



1. Effect of colchicine and cytochalasin B on Con A capping in polymorphonuclear leukocytes (PMN), il monocytes (PM), and alveolar macrophages (AM). Polymorphonuclear leukocytes, peritoneal monocytes, olar macrophages (2×10^6 cells) were preincubated at 37° in 1 ml of Krebs–Ringer phosphate buffer with ontrol (CN), 10^{-5} *M* colchicine (COL), 10 μg/ml cytochalasin B (CB), or cytochalasin B for 10 min followed ldition of colchicine (CB, COL). After 15 min, the cells were stained with FITC–Con A (50 μg) at 37°C for ien fixed in 2% formaldehyde for 10 min at 28°. The cells were washed and then pelleted and slides were m the cell button. The results were obtained by examining at least 200 cells per slide at random with a Zeiss nce microscope (490-nm wide-band interference primary filter, 530-nm secondary filter) and classifying as capped or not capped. Values represented mean ±SD of six separate experiments.

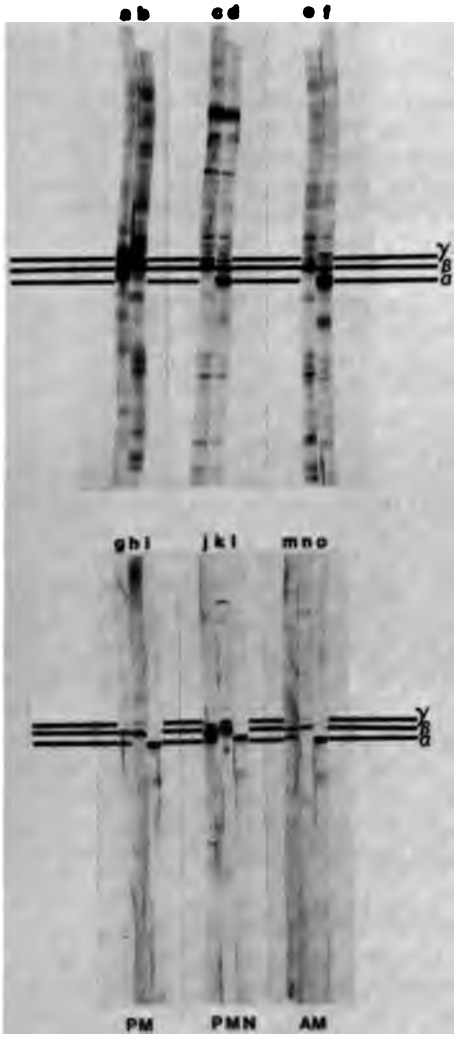


FIG. 2. Isoelectric focusing patterns of total homogenates and DNase purified actins from PMN, PM, and AM. In gels b, c, and e, 30 μg of a SDS-solubilized homogenate from each of the cells indicated was applied to polyacrylamide gels (length 13 cm) containing urea and Triton X-100 with focusing carried out 9000–10,000 V-hr. In gels a, d, and f, 10 μg of murine skeletal muscle actin (α-actin) was mixed as an internal standard with the various homogenates. Gels h, k, and n contain DNase purified actin (5–7 μg) from each cell type as indicated, while 3 μg of murine skeletal muscle actin was included in gels g, j, and m. Gels i, l, and o each contain 3 μg of skeletal muscle actin alone.

peritoneal monocytes, colchicine-induced capping was significantly attenuated by preincubation with 2-deoxyglucose, a glycolytic inhibitor, while cyanide, an inhibitor of oxidative phosphorylation, had only minimal effect on these two cells which are both dependent upon glycolysis for their major source of energy. In contrast, cyanide completely abolished colchicine-induced capping in the alveolar macrophage, a cell dependent upon oxidative phosphorylation for energy, while 2-deoxyglucose had a much less dramatic response in these cells.

Discussion. The major findings in this study were that the primary metabolic pathway employed for energy production by each cell line correlated with the response of the respective phagocytic cell to cytochalasin B-induced capping. On the other hand, there was no difference observed in the isoactin forms seen in homogenates from each cell type. Thus, by these criteria, the results suggest that the observed differences in capping properties among the cells were not due to qualitative variation among the actin forms; rather the capping response in the alveolar macrophage may depend upon a unique topographical relationship of microfilaments to the membrane (4) which may be amenable to perturbation by altering the metabolism of the phagocytic cells as has been seen in virally infected or transformed cells (12, 13). The biological basis of capping in cells of various types is not known, but differences in this parameter such as those shown here may ultimately be related to cell differentiation and metabolism.

Summary. Colchicine induced Con A capping in polymorphonuclear leukocytes, peritoneal monocytes, and alveolar macrophages, which was prevented in the polymorphonuclear leukocyte and peritoneal monocyte by cytochalasin B. Isoelectrophoresis of whole cell homogenates and purified cytoplasmic actin revealed β- and γ-actin to be present in similar amounts in all three cell types. Alveolar macrophages failed to cap in the presence of 1 m*M* potassium chloride whereas polymorphonuclear leukocytes and peritoneal monocytes maintained the capping response. In contrast, alveolar macrophages continued to cap during treatment with 5 m*M* 2-deoxyglucose abeit at lower levels than control; whereas in polymorphonuclear leukocytes and peritoneal monocytes, capping was substantially attenuated. These studies demonstrate the differing metabolic requirements

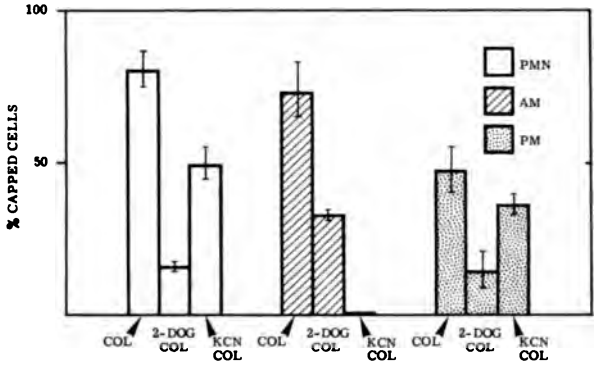


FIG. 3. Effect of metabolic inhibitors on colchicine-induced Con A capping in PMN, PM, and AM. Cells were preincubated for 45 min in either 1 m*M* potassium cyanide (KCN) or 5 m*M* 2-deoxyglucose (2-DOG) before addition of 10^{-5} *M* colchicine. After 15 min, cells were treated as in Fig. 1. All values represented mean ± SD of three separate experiments.

for capping in phagocytic cells and suggest that different topographical relationships of actin-microfilaments to the plasma membrane might exist in the alveolar macrophages compared to the phagocytic cells dependent on anaerobic metabolism.

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Prolactin-Lowering Effect of Nomifensine in the Rat (40614)[1]

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It is controversial whether in the mammalian central nervous system (CNS) there exists a distinct peptidergic prolactin (PRL)-inhibiting factor (PIF) whose secretion is under dopaminergic control, or the PIF activity of the hypothalamus may be accounted for by brain dopamine (DA) (1, 2). Dopamine released from tuberoinfundibular dopaminergic nerve terminals into the hypophysial portal circulation (3) may act directly at the level of the anterior pituitary (AP), where receptor sites for DA have been located (4). Therefore, the study of the PRL-lowering effect of drugs capable of enhancing dopaminergic neurotransmission without directly affecting DA receptor sites located on the AP may be of value in elucidating the function of the "central" dopaminergic component involved in the control of PRL secretion. Nomifensine (Nom), a recently developed antidepressant drug (5), which has a pharmacologic profile somewhat different from that of the classic tricyclic antidepressants (6), appears to be a drug suitable for this purpose. Reportedly, it activates DA neurotransmission mainly by inhibiting DA reuptake (7, 8) and facilitating DA release (9), with no ability to affect directly DA receptor sites. This manuscript reports the results of experiments on the PRL-inhibiting effect of Nom in the rat.

Materials and methods. Animals. Adult male and female rats of the Sprague–Dawley (S.D.) strain (Nossan, Corezzana, Italy) weighing 250–300 g body wt were used in most of the experiments. All rats were housed in an air-conditioned room with controlled lighting (lights on 6 AM–8 PM) and fed a standard rat diet and water *ad libitum*. Rats were maintained in this environment for at least 3 days prior to experiments.

In the experiments in rats bearing an AP transplant under the kidney capsule (see below), hypophysectomized (hypox) female S.D. rats (110–130 g body wt) and intact female littermates, which served as pituitary donors, were used. Fifteen days following hypophysectomy by transauricular route (10), hypox rats received transplants equivalent to an entire AP under the kidney capsule. Following transplantation and before the experiments, animals were weighed daily; only animals which showed a constant body weight increase throughout this period were included in the study. Thirty female S.D. rats underwent bilateral ovariectomy and were used 15 days after surgery.

Experimental procedure. In vivo experiments. Experiments were performed starting at 8.30–9.30 AM or 3.00–4.00 PM (ovariectomized rats).

At different time intervals after drug treatments (see Results), rats were either killed by decapitation or sampled repeatedly by the retroorbital venous technique (11); blood was collected into heparinized tubes, plasma was then separated and stored at −20°, until assayed for PRL by radioimmunoassay (RIA) (see below). Ovariectomized (OVX) female rats were given a single injection of estradiol (E_2, 25 μg/kg body wt) and were sampled in the afternoon of the second and third post-injection days. In the experiments with the use of α-methyl-*p*-tyrosine, intact male rats were prepared with chronic jugular cannulae inserted into the right atrium and blood samples (0.3–0.4 ml) were taken from rats housed individually in small isolation cages using a procedure previously described (12).

In vitro experiments. Male rats of the S.D. strain (200–250 g body wt) were used as AP donors. Anterior pituitaries were hemisected and four hemipituitaries were placed in 2 ml of medium TC 199 (Difco Labs., Detroit, Mich). Incubation was carried out in a Dubnoff metabolic shaker under constant gassing

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% O_2–5% CO_2 at 37°. After 30 min of bation, the medium was removed and y tissue was placed in 2 ml of fresh n containing either diluent or drugs examination (see Results). After incufor 4 hr, each AP fragment was blotted r paper and weighed. The medium zen and kept at −20° until assayed for y RIA.

s. The drugs employed in the present vere: nomifensine (8-amino-2-methyl-yl-1,2,3,4-tetrahydroisoquinoline) , Hoechst, Frankfurt, West Germany), ne (Serpasil, Ciba–Geigy, Origgio, It-methyl-*p*-tyrosine methylester (α-Prodotti Gianni, Milano, Italy), a of catecholamine biosynthesis (13), ine (Farmacia Ospedale Maggiore, , Italy), and 2-Br-α-ergocryptine (CB ndoz, Milano, Italy), which was used ect stimulant of DA receptors (14).

radioimmunoassay. Prolactin was de-d by a double-antibody radioimmu-, using the method of Niswender *et* . All results were expressed in ng/ml s of the NIH standard rat PRL-RP-1, potency is 11 IU/mg. The sensitivity PRL assay is 1.0 ng/ml; intrassay lity was 5% while repeated assay of a e plasma showed an interassay vari-f 6%. To avoid possible interassay ns all samples of each experiment sayed in a single radioimmunoassay. na PRL levels were expressed as ab-alues; in the *in vitro* experiments PRL ration in the medium was expressed nl/mg fresh pituitary. In all experi-significance of differences between was calculated by Dunnett's *t* test d by ANOVA or paired Student's *t* test where applicable. A probability of $P < 0.05$ was chosen as statistically significant.

Results. PRL-lowering effect of Nom in intact male and in OVX-E_2 primed female rats. Nom administered at the dose of 10 mg/kg i.p. induced a significant decrease of baseline PRL levels in intact male rats; its effect was significantly different from that elicited by saline injection in control rats at the doses of 2.5–5.0 and 10 mg/kg i.p. (Table I).

In a second experiment the effect of Nom on the plasma PRL rise induced by E_2 in OVX rats was compared with that evoked in the same animals by CB 154. Nom at the dose of 5.0 and 10.0 mg/kg i.p. induced a significant decrease in baseline PRL levels 60 min after injection; its effect was significantly different from that elicited by saline injection in control rats at the dose of 10 mg/kg i.p. CB 154 decreased baseline PRL levels at the doses of 1.25, 2.5, and 5.0 mg/kg i.p. In spite of a consistent diminution of baseline plasma PRL in saline-treated rats, likely attributable to a spontaneous dampening of the afternoon peak of plasma PRL at 5–6 PM (16), the lowering effect of CB 154 was statistically significant at all three dose levels ($P < 0.05$ vs saline at 120 min) (Table II).

Effect of Nom on plasma PRL levels in reserpine-pretreated male rats. A single injection of reserpine (2.5 mg/kg i.p.) induced a significant rise of plasma PRL levels 18 hr later. Nom at the dose of 10 mg/kg significantly lowered baseline PRL values in diluent-pretreated animals and was able to counteract reserpine-induced hyperprolactinemia (Table III).

Effect of Nom on plasma PRL levels in reserpine-α-MpT-pretreated male rats. Administration of reserpine (2.5 mg/kg i.p.) plus

TABLE I. EFFECT OF NOMIFENSINE (Nom) ON PLASMA rPRL LEVELS IN MALE RATS

ment[a]	Doses	rPRL ng/ml[b] t_0	rPRL ng/ml[b] $t_{1\ hr}$	Δ From baseline levels
line	2 ml/kg ip	26.4 ± 4.4	25.3 ± 4.2	−1.2 ± 3.9
m	2.5 mg/kg ip	20.3 ± 3.8	12.5 ± 2.1[c]	−7.7 ± 4.4
m	5 mg/kg ip	17.2 ± 1.5	11.5 ± 1.8[c]	−5.7 ± 3.1
m	10 mg/kg ip	24.1 ± 4.4	8.8 ± 1.2[c, d]	−15.4 ± 4.7[c]

S.D. rats (250–300 g) were sampled by the retroorbital venous technique at times 0 and 1 hr after Nom ation.

es represent the mean ± SEM of seven to eight determinations.

).05 vs saline-treated animals.

).05 vs baseline values.

TABLE II. EFFECT OF NOMIFENSINE (Nom) OR CB 154 ADMINISTRATION ON PLASMA rPRL LEVELS IN E_2-PRIMED OVARIECTOMIZED RATS

Groups[a]		rPRL ng/ml[b]		
		t_0	$t_{60\ min}$	$t_{120\ min}$
Saline	(2 ml/kg ip)	517 ± 146	397 ± 86	—
Nom	(2.5 mg/kg ip)	377 ± 57	416 ± 130	—
Nom	(5 mg/kg ip)	382 ± 55	165 ± 48[c]	—
Nom	(10 mg/kg ip)	567 ± 151	81 ± 15[c, d]	—
Saline	(2 ml/kg ip)	1149 ± 185	—	601.0 ± 105[c]
CB 154	(1.25 mg/kg ip)	476 ± 110[d]	—	38.8 ± 9.6[c, d]
CB 154	(2.5 mg/kg ip)	579 ± 112[d]	—	78.8 ± 37.1[c, d]
CB 154	(5 mg/kg ip)	521 ± 121[d]	—	59.3 ± 26.9[c, d]

[a] Ovariectomized S.D. rats (180–200 g) were given a single injection of E_2 (25 μg/kg s.c.) 15 days after surgery. Experiments were performed in the same animals starting at 3–4 PM of the second (Nom) and third (CB 154) day after E_2 injection. Rats were sampled by the retroorbital venous techniques immediately before and 1 (Nom) or 2 hr (CB 154) after drug administration.

[b] Values represent the mean ± SEM of six determinations.

[c] $P < 0.05$ vs baseline (paired t test).

[d] $P < 0.05$ vs respective saline.

TABLE III. EFFECT OF NOMIFENSINE (Nom) ON PLASMA rPRL LEVELS IN MALE RATS PRETREATED WITH RESERPINE

Pretreatment[a]		Treatment[a]		rPRL ng/ml[b]
Saline	(2 ml/kg i.p.)	Saline	(2 ml/kg i.p.)	23.1 ± 5.2
Reserpine	(2.5 mg/kg i.p.)	Saline	(2 ml/kg i.p.)	47.5 ± 7.4[c]
Saline	(2 ml/kg i.p.)	Nom	(10 mg/kg i.p.)	4.2 ± 1.8[c]
Reserpine	(2.5 mg/kg i.p.)	Nom	(10 mg/kg i.p.)	11.4 ± 1.9[d]

[a] Male S.D. rats (200–220 g) were pretreated 18 hr before Nom administration with reserpine or saline. Rats were sampled by the retroorbital venous technique 1 hr after Nom administration.

[b] Values represent the mean ± SEM of nine determinations.

[c] $P < 0.05$ vs saline-pretreated–saline-treated animals.

[d] $P < 0.05$ vs reserpine-pretreated–saline-treated animals.

α-MpT (200 mg/kg i.p.) resulted in a striking rise in baseline PRL levels which was present up to 90 min. The PRL-lowering effect of Nom, which was evident 60 and 90 min post-injection in the diluent-pretreated rats, was completely suppressed in reserpine-α-MpT-pretreated rats (Table IV).

Effect of Nom in hypox rats bearing an ectopic AP. From the results reported in Table V, it appears that administration of Nom at two dose levels, i.e., 5 and 10 mg/kg i.p., did not significantly diminish baseline PRL values 1 and 2 hr later. It must be noted, however, that in both experiments there was a trend toward lower PRL levels following administration of the higher dose of the compound.

Effect of Nom on pituitary PRL release in vitro. In a final experiment, the effect of two different doses of Nom was evaluated on the secretion of PRL from *in vitro* AP fragments and compared to that of equimolar doses of DA, a drug previously shown capable of inhibiting PRL release from *in vitro* AP (17). Nom was completely ineffective in inhibiting PRL release at both dose levels (5×10^{-7} and 5×10^{-6} *M*), whereas DA induced already its maximum inhibitory effect at the dose of 5×10^{-7} *M* (Table VI).

Discussion. Nomifensine effectively inhibited PRL release in animals with either basal or stimulated PRL secretion. This effect, likely resulting from nomifensine-induced activation of dopaminergic neurotransmission (7–9), is consistent with the reported PRL-lowering effect of dopaminergic agonists, such as bromocriptine (18), apomorphine (19), lisuride (16) piribedil (20), and the DA precursor, L-dopa (21). The PRL-lowering effect of the drug was rather slight in male rats in the unstimulated state since only the dose of 10 mg/kg was effective. In the same animal model bromocriptine strikingly lowers baseline PRL levels at the dose of 1 mg/kg (22). Instead, the PRL-suppressive effect of nomifensine was more clear cut

TABLE IV. EFFECT OF NOMIFENSINE (Nom) ON PLASMA rPRL LEVELS IN MALE RATS PRETREATED OR NOT WITH RESERPINE AND α-METHYL-P-TYROSINE (α-MpT)

Pretreatment[a]	Treatment[a]	rPRL ng/ml[b]					
		$t_{-45\ min}$	$t_{-30\ min}$	t_0	$t_{30\ min}$	$t_{60\ min}$	$t_{120\ min}$
ne	Saline						
ml/kg i.p.)	(1 ml/kg i.v.)	26.3 ± 7.1	23 ± 6.0	19.4 ± 8.5	21.4 ± 8.4	14.6 ± 4.3	20.6 ± 5.0
erpine + α-MpT	Saline						
5 mg/kg i.p.)	(1 ml/kg i.v.)						
00 mg/kg i.p.)		66.0 ± 15.0	81.7 ± 8.4	123.7 ± 18.5[d,e]	176.0 ± 28.0[d,e]	159.0 ± 18.4[d,e]	120.0 ± 19.4[d]
ne	Nom						
ml/kg i.p.)	(10 mg/kg i.v.)	40.3 ± 15.0	22.8 ± 3.6	18.4 ± 8.8	7.8 ± 2.6	5.6 ± 1.8[c]	2.9 ± 1.8[c]
erpine + α-MpT	Nom						
5 mg/kg i.p.)	(10 mg/kg i.v.)						
00 mg/kg i.p.)		69.0 ± 12.7	95.5 ± 23.0	137.0 ± 32.0[d,e]	114.0 ± 29.4[c]	116.0 ± 25.0[d,e]	118.0 ± 25.0[d]

[a] Male S.D. rats (250–280 g) permanently cannulated in the jugular vein were sampled at different times before and after Nom or saline administration. n was administered at t_0, reserpine 18 hr, and α-MpT 30 min before Nom injection.

[b] Values represent the mean ± SEM of six to eight determinations.

[c] $P < 0.05$ vs baseline values (paired t test).

[d] $P < 0.05$ vs saline-pretreated-saline-treated animals at the corresponding time.

[e] $P < 0.05$ vs saline-pretreated-Nom-treated animals at the corresponding time.

TABLE V. EFFECT OF NOMIFENSINE (Nom) ON PLASMA rPRL LEVELS IN HYPOPHYSECTOMIZED RATS BEARING AN ECTOPIC PITUITARY

Groups[a]	rPRL ng/ml[b]		
	t_0	$t_{1\ hr}$	$t_{2\ hr}$
Expt I			
Saline (2 ml/kg i.p.)	59.0 ± 17.8	54.5 ± 15.3	58.2 ± 21.9
Nom (5 mg/kg i.p.)	79.5 ± 30.6	63.2 ± 18.3	49.5 ± 21.1
Nom (10 mg/kg i.p.)	122.5 ± 47.6	48.2 ± 13.5	35.5 ± 6.2
Expt II			
Saline (2 ml/kg i.p.)	43.0 ± 12.7	44.5 ± 14.5	45.6 ± 14.5
Nom (5 mg/kg i.p.)	47.0 ± 17.2	24.1 ± 6.4	23.1 ± 7.3
Nom (10 mg/kg i.p.)	66.3 ± 39.2	18.8 ± 2.6	19.3 ± 3.8

[a] Female S.D. rats (110–130 g body wt) received an entire AP fragment under the kidney capsule 15 days before.

[b] Values represent the mean ± SEM of six to eight determinations. Rats were sampled by the retroorbital venous unique immediately before and 1 and 2 hr after Nom administration.

en baseline PRL levels had been raised ificially, i.e., in the OVX-rats given estro-ıs. In view of the reported mechanism of ion of the drug (7–9), it may be proposed .t in OVX-E_2-primed rats, despite the pres-ɔe of hyperprolactinemia, activation of DA ıırotransmission is nevertheless competent suppress PRL secretion.

In keeping with the report of Horowski d Gräf (23), who demonstrated that the .L-inhibiting effect of another indirect DA ɔnist drug, i.e., amphetamine, is main-ned in reserpine-pretreated rats, nomifen-e lowered plasma PRL when given in an-als pretreated with reserpine. This finding ıt variance with behavioral studies showing ıt the same dose of reserpine, i.e., 2.5 mg/ , completely antagonized the stereotyped king/biting activity of nomifensine (9), al-ɔugh that due to amphetamine was main-tained (24).

TABLE VI. EFFECT OF NOMIFENSINE (Nom) OR DOPAMINE (DA) ON rPRL RELEASE FROM *IN VITRO* PITUITARIES

Groups[a]	Doses (M)	rPRL ng/ml/mg pituitary
Control	—	203.1 ± 28.3
Nom[b]	5×10^{-7}	158.4 ± 25.5
Nom	5×10^{-6}	207.0 ± 8.1
DA	5×10^{-7}	93.2 ± 16.2[c]
DA	5×10^{-6}	84.0 ± 12.7[c]

[a] Each group represents the mean of five samples. For each sample four hemipituitaries incubated for 4 hr in 2 ml of TC 199 with or without drugs were used.

[b] As diluent for Nom a solution of HCl 2 N diluted 1:10.000 with TC 199 was used.

[c] $P < 0.05$ vs control.

In our study, inhibition of catecholamine synthesis in reserpine-treated animals by α-MpT abolished the PRL-lowering effect of nomifensine. In view of the different mecha-

nisms whereby reserpine and α-MpT affect brain DA stores (25, 26), these data would indicate that nomifensine causes the release of DA from a newly synthesized pool of the neurotransmitter. The same conclusion has been recently reached by Gudelski and Porter (27) for amphetamine, in experiments involving direct measurement of DA in pituitary stalk plasma.

A presynaptic action of nomifensine is also implied by its failure to inhibit PRL release from AP fragments *in vitro* and to lower the high baseline PRL levels of hypox rats bearing an ectopic pituitary. It is a common notion in fact that in mammals the AP does not contain the presynaptic neuronal component (28), through which activation of the postsynaptic catecholamine receptor occurs physiologically. However, analysis of the data reported in Table V reveals a trend toward lower PRL levels in the hypox-transplanted rats given the highest drug dose. This might imply a direct, though minor, action of the drug or a drug metabolite (29) on pituitary postsynaptic DA receptors, which in a gland disconnected from the CNS are exquisitely sensitive to dopaminergic stimulation (30, 31). Alternatively, nomifensine by acting on peripheral sites might have induced an increase in DA arterial blood levels.

The central stimulant effects of nomifensine in rodents have been causally related to the very potent inhibitory effect of the drug on the uptake mechanism in DA synaptosomes (7, 8). The present study points to a more significant role for the DA-releasing action of nomifensine. In fact, if one admits that the drug action with respect to PRL secretion is exerted at the level of the median eminence (ME), it appears unlikely that nomifensine may act to inhibit the uptake of DA which is released into the portal circulation. It is conceivable in fact the presynaptic capture of the released amine may not play at ME level a major role in the removal of the neurotransmitter, a task which is better accomplished by the downward flow of the portal blood. However, the possibility cannot be ignored that nomifensine may facilitate DA neurotransmission by blocking DA uptake at a central synapse between a dopaminergic neuron and another neuron (PIF?) of unknown nature on which postsynaptic DA receptors are located. Direct measurements of DA in portal blood before and after nomifensine treatment are needed to resolve this problem.

The availability of drugs such as nomifensine, whose action rests on the integrity of dopaminergic transmission in the CNS, offers a valid pharmacologic means to probe hyperprolactinemic states due to an altered CNS control. In this vein, results obtained in hyperprolactinemic subjects have shown that nomifensine is capable of lowering plasma PRL in "functional" hyperprolactinemia but fails to do so in patients bearing PRL-secreting adenomas (23). The dopaminergic defect present in the CNS of the tumor subjects (33, 34) is likely the cause of such behavior.

Summary. The effect of nomifensine (Nom), a new antidepressant agent which facilitates dopaminergic neurotransmission by blocking the reuptake of dopamine (DA) into nerve terminals and by promoting DA release, was studied on plasma prolactin (PRL) levels in the rat. Nom reduced baseline plasma PRL levels in untreated or reserpine-pretreated male rats and in ovariectomized, estrogen-primed female rats. Nom did not alter plasma PRL levels in reserpine-α-methyl-*p*-tyrosine-pretreated male rats and in hypophysectomized female rats bearing a pituitary transplant under the kidney capsule nor inhibited PRL release from *in vitro*-incubated pituitaries. The availability of drugs such as Nom, whose action rests on the integrity of dopaminergic transmission in the CNS, without involving direct stimulation of pituitary DA receptors, offers a valid means to investigate CNS function in hyperprolactinemic states.

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Bacterial Stimulation of Serum Colony-Stimulating Activity and Neutrophil Production in Germ-Free Mice (40615)[1]

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The factors regulating granulocyte production are poorly understood. The most likely humoral regulator of granulocytic production and maturation is called colony-stimulating factor (CSF) (1). The ability to stimulate granulocyte colony growth *in vitro* is termed colony-stimulating activity (CSA) (2, 3). The major source of CSA in humans appears to be the monocyte–macrophage system (4–8), although lymphocytes have also been implicated in CSA production (9, 10). The factors which modulate CSA production or release appear to be, in part, the result of stimulation by microorganisms and their products, particularly endotoxin. Previous studies have shown that incubation of monocytes with whole live or killed bacteria, or with endotoxin, enhances or increases CSA production by these cells (11–14). Likewise, it has been shown that injection of endotoxin into humans or mice leads to increased levels of serum CSA (15–17).

The role of mature peripheral blood and tissue neutrophils in regulation of their own production is open to controversy. Some authors have suggested that mature neutrophils may make substances which inhibit new granulocyte production and maturation, particularly when they are present in increased numbers (18–20). Recent studies have suggested that neutrophils may modulate their own production by inactivation of microorganisms and their products, thereby negating their stimulatory effect on CSA production (13, 14). The present study was undertaken to investigate further the role of microorganisms and blood neutrophils in the modulation and regulation of CSA production. Changes in serum CSA levels, bone marrow colony-forming cells (CFU-C), absolute granulocyte counts, and bacterial levels in germ-free mice after introduction of bacteria into their environment were investigated. These studies have shown that a marked rise in CSA production occurred immediately after germ-free mice were moved into a nonsterile environment. This rise in CSA was correlated with a rise in stool bacteria levels and was followed by a rise in the absolute granulocyte count. As the absolute granulocyte count rose, both CSA production and stool bacterial levels decreased. These studies are compatible with the suggestions that CSA production is modulated by the levels of microorganisms and their products and that neutrophils serve as a negative feedback arm in the scheme of granulocyte regulation by inactivation of the stimulatory effect of microorganisms.

Materials and methods. Forty-two-day-old Swiss–Webster axenic mice obtained from Charles Rivers Laboratories were used in these experiments. Animals were maintained in a germ-free state in cages provided by the supplier for 24 hr and then moved to a filtered air environment and fed sterile Purina Mouse Chow. They were given 1×10^8 *Escherichia coli* per animal orally at this time. Five mice were sacrificed for each time point prior to, and after movement into, the filtered air environment and introduction of *E. coli*. At sacrifice, blood was collected by cardiac puncture for white blood cell counts, differential counts, and serum CSA values. Stools were collected for measurement of bacteria levels, and bone marrow was obtained for quantitation of CFU-C.

CSA determinations. Serum CSA was determined by methods previously described

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:rum (0.1 ml) from a pool of five test
)r each time point was plated into
te 35-mm plastic petri dishes to which
ded a 1-ml mixture of 0.5% McCoy's
lium containing 15% fetal calf serum.
were then overlayed with 75,000 nu-
cells derived from the femurs of C57/
:e suspended in 1 ml of McCoys with
gar. After gelling, the plates were in-
l in a fully humidified incubator with
tant flow of 7.5% CO_2 in air. The
stimulus for colony growth was a
d human urine. After 7 days of incu-
colonies were counted using a stereo
)ope. Only aggregates containing 50 or
:lls were scored as colonies.

ny-forming unit determinations. CFU-
n the femurs of the experimental mice
easured by isolation of a single femur
ıch animal and the marrow was blown
he shaft using a syringe containing
's 5A medium. A single cell suspen-
as prepared, followed by a total cell
Nucleated cells (75,000) were then
in a 1-ml aliquot of McCoy's 5A me-
)ntaining 0.3% agar and 15% fetal calf
The stimulus for colony growth was
l of a standard human urine. The
were incubated as above and counted
days.

rmination of bacterial levels. After sac-
he large intestine of the experimental
was opened and a single formed fecal
was removed, weighed, and incubated
ndard amount of trypticase soy broth
hr. The number of bacteria were
l in a hemocytometer and also plated
ine laboratory fashion on a standard
ate followed by colony counts after 24
hr.

lts. Figure 1 shows the correlation be-
serum CSA values, fecal bacterial lev-
d absolute granulocyte counts in ex-
ntal mice before and after removal
ieir germ-free environment and intro-
ı of bacteria. Absolute granulocyte
and serum CSA values were low in
m-free state when compared with val-
)orted in conventional mice (22, 23).
)wer range levels were again attained
7 after the introduction of bacteria as
:e returned to the germ-free state. No
a were detected in fecal specimens.

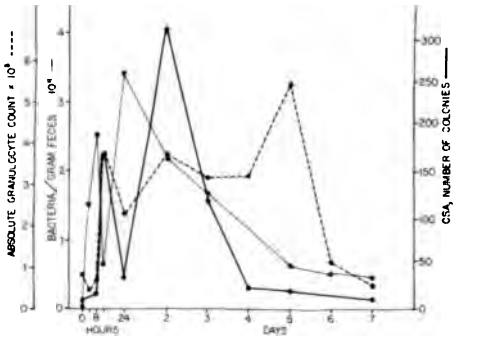

FIG. 1. Changes in serum CSA values (——), fecal bacterial levels (·····), and absolute blood granulocyte counts (– – –) in germ-free mice before and after removal from germ-free environment and oral bacteria introduction. CSA values are the mean colony numbers stimulated of 0.1 ml of pooled sera from five mice. Fecal bacteria levels are expressed as colonies per gram of feces cultured. Time 0 is the point of removal from germ-free environment and introduction to oral bacteria.

Soon after introduction of bacteria and removal from the germ-free environment, there was an initial drop in absolute granulocyte counts at Hour 4 as fecal bacterial levels rose. As fecal bacterial levels continued to rise, so did serum CSA values and absolute granulocyte count. After 2 days, 36 hr following the initial serum CSA rise, there was a second peak in absolute granulocyte counts. The maximum increase in serum CSA values was seen at Hour 48 and this was followed 72 hr later, on Day 5, by a dramatic rise in absolute granulocyte counts. This rise in absolute granulocyte counts was correlated with a concomitant fall in fecal bacterial levels and serum CSA values. The peak of granulocytes was preceded by the commencement of the fall in CSA and in bacteria.

Table 1 shows the correlation between bone marrow CFU-C levels, serum CSA values, absolute granulocyte counts, and fecal bacterial content in these same animals. It should be noted that the standard deviations reflect the reproducibility of the assay, not variation from animal to animal, since pooled specimens were used. The initial rise in serum CSA values at Hour 12 following introduction of bacteria and removal of these animals from their germ-free environment is correlated with a fall in bone marrow CFU-C levels at Hour 8. This fall in bone marrow CFU-C levels is, in turn, correlated with an

TABLE I. LEVELS OF BACTERIA, GRANULOCYTES, CSA, AND CFU-C BEFORE AND AFTER ADMINISTRATION OF *E. coli* TO GERM-FREE MICE[a]

Time after administration of *E. coli*	No. of bacteria per gram feces ($\times 10^{11} \pm$ SD)	Absolute granulocyte count ($\times 10^3 \pm$ SD)	CSA (No. of Colonies ± SD)	CFU-C per femur ($\times 10^3 \pm$ SD)
Preadministration	0 —	0.81 ± 0.21	7 ± 01.5	10.60 ± 1.70
4 hr	1.68 ± 0.20	0.45 ± 0.13	—	7.09 ± 0.68
8 hr	2.42 ± 0.09	0.69 ± 0.07	15 ± 7	3.36 ± 0.64
12 hr	0.61 ± 0.20	3.62 ± 0.67	168 ± 8	4.05 ± 1.20
24 hr	3.35 ± 0.36	2.36 ± 0.35	31 ± 10	13.03 ± 0.77
2 days	2.10 ± 0.17	3.75 ± 0.72	309 ± 9	17.2 ± 1.40
3 days	1.61 ± 0.15	3.18 ± 0.05	117 ± 1	24.18 ± 0.99
4 days	—	3.23 ± 0.46	19 ± 4	13.81 ± 1.50
5 days	0.48 ± 0.11	5.39 ± 0.08	17 ± 5	18.81 ± 2.01
6 days	0.37 ± 0.02	1.15 ± 0.45	—	14.54 ± 0.12
7 days	0.437 ± 0.04	0.68 ± 0.36	13 ± 5	6.89 ± 0.65

[a] The mice were administered 1×10^8 organisms per animal. At each time point, animals were sacrificed and levels of bacteria, granulocytes, CSA, and CFU-C were assayed. Results (for each time point) are the mean of five animals.

initial rise in absolute granulocyte counts seen at Hour 12. The CFU-C levels continued to rise, but once the maximum rise in serum CSA values had occurred, bone marrow CFU-C levels gradually returned to lower levels over the following 5-day period. The absolute granulocyte counts and serum CSA values also returned to normal levels following the dramatic rise in absolute granulocyte counts on Day 5.

Discussion. There seems little doubt that granulocyte production must be associated with the levels of microorganisms and their products *in vivo*. Any increase in microorganism load over the normal endogenous state leads to a prompt increase in granulocyte production to combat this insult. The mechanism whereby this occurs has previously been unclear. The data presented here, and in previous studies, suggest that this is the result of a stimulatory effect of microorganisms and their products on the factors regulating granulocyte production, maturation, and release (12, 13, 15–17). These data suggest that peripheral blood granulocytes serve a role in regulating their own production by inactivating microorganisms, specifically bacteria, thereby decreasing the production of granulocyte stimulatory factors and, hence, their own production.

Previous studies from this laboratory of patients undergoing bone marrow transplantation have suggested that similar mechanisms may be operative in humans (24). In these studies it was shown that serum CSA values rise dramatically as patients become profoundly granulocytopenic and return to low or normal levels as bone marrow engraftment ensues and the absolute granulocyte counts rise. It was shown in these studies that there was a direct correlation between rises in serum CSA levels and fever curves. In these initial human studies no measurement of bacterial content to correlate with the fever curves and CSA values were carried out. These data might suggest that the microorganism load may have accounted for the rises in temperature and serum CSA values noted in these patients.

Further support for such a mechanism is obtained from the studies of Quesenberry *et al.* (25), who studied the changes in serum CSA values of conventional and germ-free mice rendered neutropenic by whole body irradiation and demonstrated that dramatic rises in serum CSA values occurred in conventional animals with the development of neutropenia, but no such rise was found in germ-free animals, despite similar falls in peripheral blood neutrophil levels. They suggested that the factors necessary to stimulate CSA production were absent in germ-free animals and that peripheral blood neutrophil levels alone were not the major determining or modulating force for CSA production.

Other authors have suggested that mature circulating and tissue neutrophils may produce factors which inhibit their own production and maturation. These factors have been sometimes called chalones (18, 26) but con-

evidence for a physiologic role of such
ials is, at best, presumptive.
prefer to view neutrophil regulation
a more physiologic standpoint. In the
ıe presented previously (14, 27), we have
sted that the major positive or stimula-
force for neutrophil production is the
of microorganisms and their products
erhaps other antigenic stimuli, and the
ive feedback arm inactivation of micro-
isms and their products by mature neu-
ils. The present data seem in concert
his concept.

nmary. The *in vivo* effect of microorga-
upon granulopoiesis of axenic mice was
d. A marked rise in CSA production
red immediately after germ-free mice
exposed to microorganisms. This rise in
was correlated with a rise in stool bac-
levels and a rise in absolute granulocyte
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decreased. The peak of granulocytes
eached after the commencement but
g the fall of CSA and bacteria levels.
studies are compatible with the sugges-
hat CSA production is modulated by
vels of microorganisms and their prod-
and that neutrophils serve as a negative
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ation by inactivation of the stimulatory
of microorganisms.

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A Soluble Bone Morphogenetic Protein Extracted from Bone Matrix with a Mixed Aqueous and Nonaqueous Solvent (40616)[1]

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Development of bone occurs in intramuscular or subcutaneous implants of dentin (1, 2) and bone (3–6) matrix in postfetal life in an extraskeletal site under the influence of a bone morphogenetic protein (BMP). BMP is transferred from bone matrix to a responsive mesenchymal-like cell population within 24 hr after implantation (7). BMP is bioassayed by measurements of the quantity of histologically valid new bone per unit weight of preimplanted matrix 28 days after the operation (8). At earlier intervals, BMP activity can be estimated by the accumulation of the following specialized cell products in the implant: hyaluronate within 24 hr (9); hyaluronidase within 48 hr (10); ^{35}S-sulfated substances within 7 days (11); alkaline phosphatase within 10 days (12); ^{45}Ca uptake by mineralizing tissue within 10 to 14 days (13). All of these data on BMP activity are based on observations on implants of demineralized bone matrix or of insoluble bone matrix gelatin (14–18). Recently, with the aid of a modification of the methods of Herring *et al.* (19, 20) and Ashton *et al.* (21), we separated a soluble BMP from bone matrix digested with purified bacterial collagenase (22). We report here on a method of solubilization of BMP without digestion of the bone matrix.

Materials and methods. Eighty grams of rabbit cortical bone was excised from the midshaft of the long bones, pulverized manually under aseptic conditions with a mallet, washed 3× in 1 liter of cold distilled water to remove the old blood and bone marrow, and then demineralized in cold 0.6 *N* HCl within a period of 24 hr. After the acid was removed by dialysis against 5 mmol/liter of NaN_3 at 2°, the demineralized fragments were extracted in a solution of 50% ethylene glycol (EG) in 0.05 *M* phosphate-buffered 0.15 *M* sodium chloride (EG-PBS) at 25° as outlined in five steps shown in Fig. 1. The supernatant solution was separated from the matrix powder by centrifugation at 40,000g for 30 min, decanted, and desalted by dialysis against 0.01% EG in cold distilled water in membrane tubing (Spectrophor, MW 2000). The nondialyzable desalted substances were lyophilized, and analyzed by standard methods for hydroxyproline (23), hexosamine (24), total phosphorus (25), total Lowry protein (26), and amino acids (27). The experiment was repeated substituting 0.025 *M* Tris buffer for phosphate buffer to avoid phosphate binding and eliminate artifacts from measurements of total phosphorus.

For controls, EG-PBS extracts were similarly prepared from bone autolyzed (28) or denatured by demineralization in a solution of 70% alcohol in 0.6 *N* HCl to abolish matrix BMP activity (4).

The substances in solution obtained by Step 5 (Fig. 1) were dialyzed for 8 hr first against 100 mmole/liter of calcium chloride, then against phosphate buffer for 24 hr pH 7.2, to produce a protein calcium phosphate coprecipitate. The coprecipitate was washed three times in a 0.1% EG solution at 2°, in cold water and lyophilized. The protein was recovered by mechanical disaggregation and extraction of the coprecipitate with a 50% EG-PBS solution, and desalted by dialysis against 0.1% EG in cold water.

Analytical sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Samples of the lyophilized substances obtained by the procedure shown in Figs. 1 and 2 were incubated for 2 hr in 0.01 *M* sodium phosphate buffer, pH 7.0, containing 1% SDS and 1% β-mercaptoethanol prior to application to a 10% gel. Slab gel electrophoresis was carried out by the method of Weber and Osborn (29).

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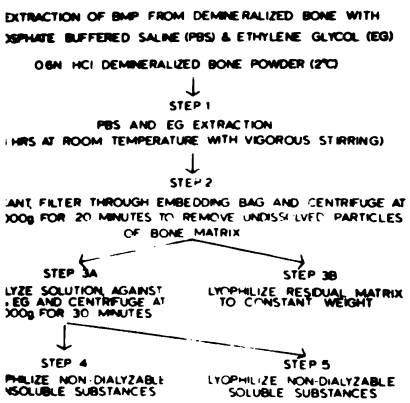
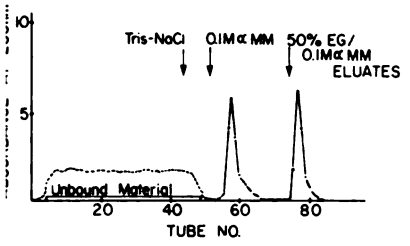


1. Flow diagram of five steps in the ethylene phosphate-buffered salt solution (EG-PBS) ex- of hydrophobic noncollagenous proteins from atrix. The dry weight of the substances obtained step is shown in Table I.



2. Chromatography of EG-PBS solution ap- a Con-A-S column and eluted in sequence with and EG. A filtered and centrifuged EG-PBS (350 ml in volume) was dialyzed against 0.05 *M* Cl (pH 7.4) in 0.15 *M* NaCl and applied to a S column (1.6 × 10 cm) equilibrated with the uffer. The unbound fraction contained nearly all EG-PBS-soluble collagen peptide chains and a uantity of various hydrophilic noncollagenous s. The α-MM and EG elutes contained only trace es of hydroxyproline-containing collagen pep- d significant quantities of hydrophobic glycopro-

els were stained in a 1:1 mixture of assie brilliant blue G-250 and alcian stain. For determination of apparent ular weights with the following stand- Bio-Rad Lab, Richmond, Calif.) were phosphorylase, 94,000; bovine serum in, 68,000; ovalbumin, 43,000; carbonic rase, 30,000; soybean trypsin inhibitor, ; lysozyme, 14,300.

canavalin A (Con-A-S) Sepharose chro- raphy. The soluble nondialyzable com- ts were desalted by dialysis against 5% ne glycol in water and applied to a Con-A-S (1.6 × 10-cm) column equlibrated with phosphate-buffered saline (PBS), pH 7.4, as described by Davey *et al.* (30). When the absorption of the washings dropped to almost zero, the column was eluted with 150 ml of either 0.1 *M* α-methyl mannoside (α-MM) in PBS or 50% EG until two more 280-nm absorbing components were detected. The fractions collected from each peak were lyophilized for chemical analysis.

Bioassay. Bioassays for BMP were performed by implantation of 1, 2, and 5 mg of lyophilized material obtained from Steps 3 to 5 were implanted in double-walled cellulose acetate (Millipore, 0.45-μm pore size) chambers (30). The chambers were implanted in the anterior abdominal wall of allogenic rabbits (New Zealand) for 21 days. The function of the chambers is to release the protein slowly and over a period of time sufficient for a population of mesenchymal cells to gather.

The yield was determined morphometrically by the percentage of radioopaque histologically valid deposits of new bone covering the outer membrane of each chamber in microradiographs and histological sections stained in hematoxylin eosin and azure II.

Results. Table I summarizes measurements of the quantity of various fractions obtained by EG-PBS extraction of 0.6 *N* HCl demineralized bone matrix and estimates of the bone morphogenetic activity associated with each fraction. The EG-PBS extract was decanted and centrifuged at 40,000*g* for 1 hr to separate insoluble from soluble substances. The sedimented material was lyophilized, weighed, and analyzed. The supernatant solution was desalted by dialysis against 0.1% EG for 24 hr and until a straw-colored precipitate formed. After centrifugation, the precipitate was washed in 0.1% EG and lyophilized. The dry weight of the precipitate was 18.0 μg per mg of lyophilized bone matrix. The 0.1% EG-soluble substances (Step 5), after lyophilization, were fluffy white and weighed 28.4 μg/mg of bone matrix. The residual matrix was washed, lyophilized, weighed, and twice reextracted with EG-PBS to remove all soluble substances and leave a residue devoid of all BMP activity.

The chemical composition of substances derived from Step 5 in μg/mg was: total protein, 200.0 ± 12.0; amino acids, 194.4 ±

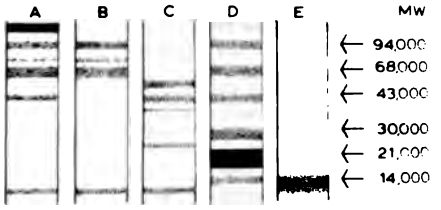
TABLE I. YIELD OF BMP AND ASSOCIATED SUBSTANCES EXTRACTED FROM BONE MATRIX WITH EG-PBS

Substances obtained by step shown in Fig. 1	Description	Yield (mg/g) dry demineralized bone matrix	Percentage of the outer membrane surface covered with new bone
4	Water insoluble, EG-PBS soluble	18.0	0
5	Soluble in 0.1% EG	28.4	34.1 ± 9.0
5 Applied to Con-A column	Unbound protein	0.027	0
5 Applied to Con-A column	α-MM eluate	0.015	15.4 ± 2.0
5 Applied to Con-A column	EG eluate	0.014	12.9 ± 2.5
5 Coprecipitated with calcium phosphate	EG-PBS extract of coprecipitate	0.001	40.5 ± 6.0

21; hydroxyproline, 0.6 ± 0.1; hexosamines, 9.1 ± 1.2; total phosphorous, 4.9 ± 0.1. The error was eliminated from measurements of total phosphorus by substituting Tris-buffered NaCl for phosphate-buffered NaCl.

Concanavalin A–Sepharose chromatography. The above-described solution was applied to a Concanavalin A–Sepharose column equilibrated with Tris-buffered 0.15 *M* NaCl at pH 7.4. When the absorption dropped to almost zero, columns were eluted with α-methyl mannoside (α-MM) or α-MM in ethylene glycol (EG) and the eluates were collected for biochemical and biologic assays. The breakthrough fraction contained 274 μg protein/mg total product, 3.2 μg/mg hexosamine and 1.4% collagenous material (estimated on the basis of hydroxyproline content). The α-MM eluate contained 220.9 μg/mg total (Lowry) protein/mg product contaminated with 0.2 μg/mg of hydroxyproline. The EG eluate contained 188 μg/mg of protein contaminated with 0.4 μg/mg of hydroxyproline (Fig. 2).

Figure 3 is a diagram of the results of slab

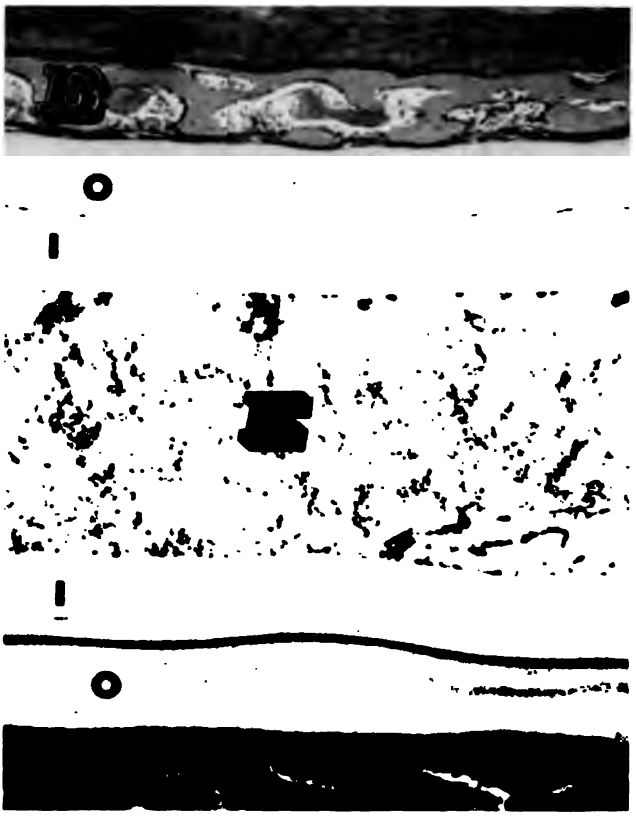


A. EG-PBS TOTAL EXTRACT
B. CON A-S CHROMATOGRAPHY-α-MM ELUATE
C. CON A-S CHROMATOGRAPHY-EG ELUATE
D. STANDARDS
E. COPRECIPITATE, EG-PBS EXTRACT

FIG. 3. Diagrammatic representation of slab SDS-acrylamide gel electrophoresis patterns of β-mercaptoethanol-reduced fractions demonstrated in Figs. 1 and 2.

SDS–gel electrophoresis of the solution obtained by Step 5, and various subfractions. The total EG-PBS extract contained not less than six components with assigned molecular weights ranging from 100,000 to 12,000. The α-MM eluate contained about four stainable components and the EG about five with electrophoretic mobilities corresponding to standards ranging from 94,000 to approximately 12,000 in MW. Electrophoretic analysis of fraction E coprecipitate (Fig. 3) shows only one band. More recent experiments, however, with larger quantites of material applied to the gel, show three additional bands corresponding to components of higher MW. Based on uv absorbance, estimates of the weight of mixture of the four components of the α-MM eluate are about 15 mg, and of the five components of the EG eluate, 14 mg (Fig. 2).

Bioassays of lyophilized substances in diffusion chambers, as noted in Table I, showed that the total EG-PBS extract, and both the α-MM, the EG eluates, as well as the coprecipitated substances all induced transmembrane new bone formation (Figs. 4 and 5). The incidences of positive results were 3/4, 2/4, 2/2, and 3/4, respectively. Implants of the EG-PBS-insoluble substances, or control PBS-EG extracts of matrix prepared from autolyzed or acid alcohol denatured bone did not induce transmembrane bone morphogenesis. Neither did the denatured collagen and other soluble proteins collected in the unbound fractions of substances separated by Con-A column chromatography.



4. Low-power magnification photomicrograph of deposits of new bone formed on both sides of a double-diffusion chamber containing 5 mg of EG-PBS-extracted bone matrix proteins 3 weeks after implantation. emnants of extract (E); inner membrane (I); outer membrane (O); new bone (B).

cussion. The working hypothesis of the -described experiments is that the BMP ydrophobic protein molecule or protein gate that is dissociated from collagen ther hydrophilic substances by the use G, a water miscible nonpolar carrier. eptually, an EG·BMP complex is stru y stable and biologically active while free in a purely aqueous medium is gically inactive. Such EG·protein com- are formed by many biologically active ns which are not bound by covalent es into some generally insoluble matrix According to Tanford (31) and Singer hydrophobic proteins are difficult to ve and may even be expelled from us solutions, yet may become miscible tain nonaqueous solvents. The solvent cause neither rupture nor formation of ovalent bonds within the macromole- ther than those within hydrogen atoms, herefore, should be chemically as inert as possible. Hydrophobic solvent mixtures sustain the biologically active conformation of proteins in aqueous media by clustering the hydrophobic amino acid components on the inside of a molecule away from the aqueous environment. In a mixture of water and EG, a weakly protic nonpolar solvent, hydrophobic interactions would protect the native conformation of BMP.

BMP·EG and other glycoprotein complexes may be bound to a Con-A column in two ways, one by carbohydrate recognition and another presumably by hydrophobic interaction. Analytical SDS–gel electrophoresis demonstrates that after mercaptoethanol reduction the α-MM-eluted fraction includes at least four while an EG eluate about five stainable bands. By comparison of the electrophoretic mobilities of the component bands with standard purified proteins, the molecular weights range from 94,000 to less than 14,000. However, these are not protein

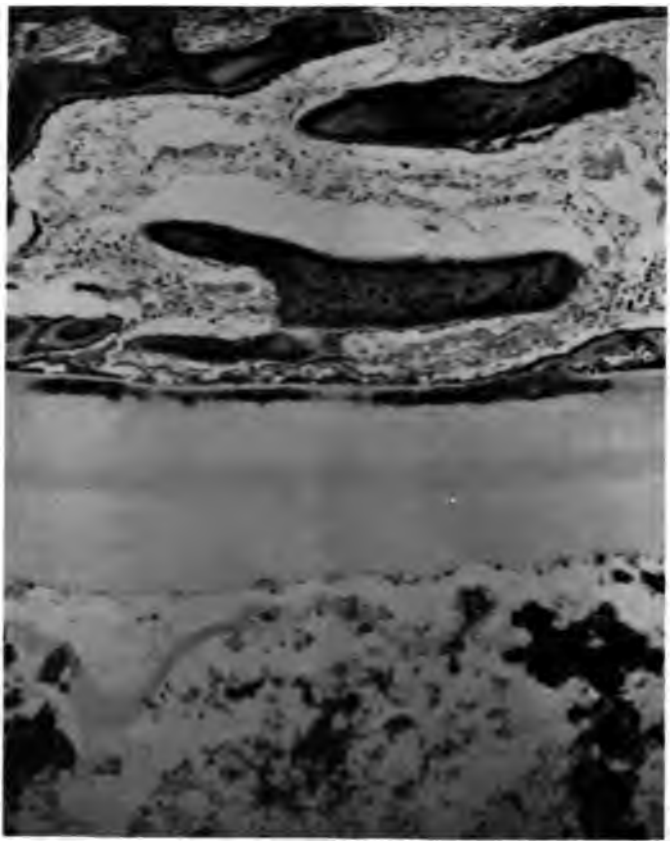


FIG. 5. High-power magnification photomicrograph of 4-week-old bone deposits (top) on the outside of diffusion chamber filled with a coprecipitate of calcium phosphate and the proteins in an EG-PBS extract (bottom). Undecalcified section, von Kossa stain.

MW estimates, but estimates based on electrophoretic mobilities of mercaptoethanol-reduced EG·protein complexes and must be corrected for the weight of EG bound to unreduced proteins by the use of D_2O and the analytical ultracentrifuge (30). Furthermore, BMP is a disulfide-bonded molecule and is reversibly inactivated by mercaptoethanol reduction and oxidation (6).

The above observations indicate the combinations of experiments remaining to be done to ascertain which of the electrophoretically resolved components might have BMP activity. The principal problem is to define the smallest complete unit of BMP structure. Present observations on the binding of BMP active components to a Con-A simply affirms the view that BMP is a hydrophobic glycoprotein (22). BMP is now selectively separated from other proteins in the same way that coprecipitation with calcium phosphate separates serum α-HS glycoprotein from albumin (33). Thus far, subfractionation of the Con-A chromatographed and of extracts of the calcium phosphate-coprecipitated proteins by Sephadex G-100 gel filtration, De-52 ion exchange, and other systems has not yet proven to isolate a BMP. The main difficulty is that each one subfraction weighed much less than 100 μg, a quantity of protein required for both bioassay and complete molecular characterization. However, to obtain the optimum quantity of starting material, we have recently substituted kilogram quantities of bovine bone for the 80-g batches of rabbit bone described in this report, and have a secured, biologically active crude bovine BMP. Bovine BMP-induced differentiation of bone in muscle pouches in both rats and rabbits. The composition of crude bovine BMP is under detailed investigation and will be reported in another communication.

nmary. A soluble bone morphogenetic n (BMP) along with other noncollage- proteins including only a relatively in- icant quantity of hydroxyproline-con- g peptides were extracted from rabbit al bone by the action of an aqueous- ueous solvent mixture. As it is bound rbohydrate recognition and hydropho- iteraction with Con A, BMP has an ial characteristic of a glycoprotein. α-methyl mannoside and ethylene gly- ites of proteins bound to a Con-A col- are implanted inside of double-walled ion chambers, deposits of cartilage and develop on the outside. While estimates quantity of BMP are less than 0.01% of y weight of bone matrix, it is possible centrate and purify a biologically active by application of the principles of hy- obic protein chemistry.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 54-57 (1979)

Mechanism of the Protective Effect of Angiotensin-Converting Enzyme Inhibitic Hemorrhagic Shock (40617)[1]

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Recent evidence indicates that angiotensin I-converting enzyme inhibitors (CEI) exert beneficial effects in cats (1) and dogs (2, 3) in hemorrhagic shock. The mechanism of the protective effect of CEI could involve actions on either of two major vasoactive peptides: (a) blockade of angiotensin II formation or (b) potentiation of vascular effects of bradykinin (4). In order to differentiate between these two potentially protective mechanisms, the following experiments were performed. Captopril, a CEI, was administered to hemorrhaged cats with and without angiotensin II infusion, thus examining the role of inhibition of angiotensin II formation on the CEI-induced shock antagonism.

Additionally, bradykinin vascular responses were examined after CEI administration in normotensive and hypotensive cats. This experiment tested whether the CEI potentiation of bradykinin was present in hypotensive states. Therefore, the aim of this report was to define the importance of blockade of angiotensin II formation to the protective effect of CEI in hemorrhagic shock, and to examine the influence of CEI on bradykinin vascular responses in hemorrhage.

Materials and methods. Male cats were anesthetized with intravenously injected pentobarbital sodium (30 mg/kg). A carotid artery and a jugular vein were catheterized for the continuous recording of arterial blood pressure and central venous pressure, respectively. A femoral artery was also cannulated to provide a route for blood removal and was attached to an oxygenated, heparinized Lucite reservoir which stored the shed blood during oligemia. A femoral vein was also cannulated for infusion of pharmacological agents. A laparotomy was performed, and a 1.5 mm (i.d.) electromagnetic flow pro placed around the superior mesenteric for blood flow measurement.

In those animals subjected to hemo shock, cats were bled to 40 mm H maintained at that pressure for 2.5 h servopump connected to the reservo shed blood remaining in the reservo then reinfused, and the animals we served for an additional 2 hr. Two gr animals were involved in this portion study: (i) hemorrhaged cats receivi CEI, and (ii) hemorrhaged cats receivi CEI in addition to angiotensin II. Ca (0.5 mg/kg) was administered imme after 0 time and was infused at a rate mg/kg/hr for the remainder of the mental period while angiotensin II v fused at 10 μg/kg/hr. This infusion angiotensin II results in a circulating tensin II concentration of 1–2 ng/ml, centration that is present in animals i orrhagic shock (1, 3).

In the second portion of the stu utilized normotensive animals (i.e., ab mm Hg) and cats bled to a mean pressure of 80 mm Hg. In both groups angiotensin I (1 μg/kg) and bradykinin kg) were injected before and at hourly vals after captopril (0.5 mg/kg) injec single injection of the CEI was give enabling observation of the rate of re from converting enzyme blockade.

Results. Mean postreinfusion survival of the two groups of hemorrhagic sho are presented in Fig. 1. The cats re only captopril exhibited a survival t 110 ± 4 min (mean ± SEM), whereas tensin II infusion (10 μg/kg/hr) in co tion with captopril resulted in a signi shorter survival time (64 ± 11 min, P < These results indicate a reversal of th induced protection in hemorrhagic sh addition of angiotensin II. Also shown

[1] Supported in part by a grant from the W. W. Smith Foundation.

[2] Fellow of the Ischemia-Shock Research Center.

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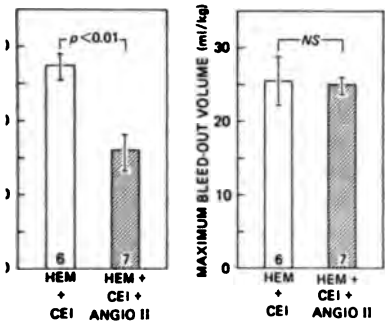

. Survival times and maximum bleedout vol-
:ats in hemorrhagic shock. The prolonged sur-
e in CEI-treated animals was significantly re-
' infusion of angiotensin II. Although mean
ime was reduced, the severity of hemorrhage
'alent in both groups as indicated by the similar
1 bleedout volumes. Numbers within the bars
the number of cats in each group; brackets
SEM.

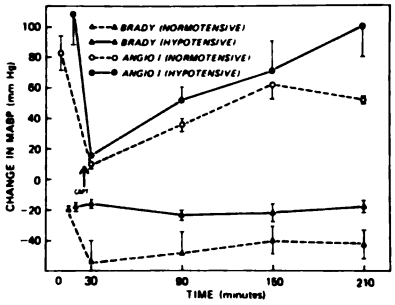

FIG. 2. Mean arterial blood pressure (MABP) responses to angiotensin I (1 μg/kg) and to bradykinin (1 μg/kg) prior to and following CEI administration. Angiotensin I responses were significantly attenuated after injection of CEI in normotensive and hypotensive animals. Bradykinin responses were significantly enhanced in normotensive but not in hypotensive cats. Both groups consisted of five cats. The arrow at 30-min marks the time of captopril (CAPT) (0.5 mg/kg) injection. Values are means ± SEM.

ıe maximum bleedout volumes of the oups. Maximum bleedout volumes rtually identical, indicating that both were subjected to an equivalent sever- emorrhagic shock.

rior mesenteric artery flows (SMAF))mparable initially in the two hemor- groups (i.e., 10.6 vs 13.6 ml/kg/min). roups of cats experienced similar flow ons during the oligemic period. At the oligemia SMAF were 2.4 ± 1.0 vs 1.9 nl/kg/min. However, cats given only ril developed significantly higher lows immediately following reinfusion) ± 1.9 vs 2.6 ± 1.0; $P < 0.001$). These ggest a susceptibility of the splanchnic ıture to angiotensin II following rein-)f shed blood. Moreover, the improved .l time of animals given captopril may ted to the improved splanchnic perfu- served in response to this CEI.

n arterial blood pressure (MABP) s in response to angiotensin I (1 μg/ l bradykinin (1 μg/kg) are summarized 2. Prior to CEI administration (i.e., at ', angiotensin I increased blood pres- ± 14 and 108 ± 20 mm Hg in nor- ive and hypotensive cats. Immediately EI injection, these responses were re- :o 10 ± 3 and 16 ± 2 mm Hg, respec- During the following 3 hr these pressor responses gradually increased to control levels, presumably due to degradation of the CEI. Bradykinin depressor responses, conversely were significantly enhanced by the CEI, from −20 ± 2 to −54 ± 14 mm Hg in normotensive cats. This potentiation of the bradykinin responses diminished slightly with time, but was not totally eliminated at 210 min. In contrast to these results, bradykinin responses in hypotensive cats were not altered by the CEI, indicating a lack of CEI potentiation of the depressor effect of bradykinin at low arterial blood pressures such as those existing in hemorrhagic shock.

Superior mesenteric artery flow (SMAF) responses to angiotensin I and bradykinin are summarized in Fig. 3. SMAF responses to angiotensin I were significantly reduced from −55 to less than −15% in both groups of cats following administration of the converting enzyme inhibitor. These flow responses returned to control values at 210 min. The bradykinin-induced increases in SMAF were only moderately potentiated by the CEI in normotensive animals (+52 vs +84%), but did not occur in the SMAF response in hypotensive cats. This lack of potentiation of the splanchnic artery dilation to bradykinin by captopril in hypotensive cats is consistent with the blood pressure results. Together these data indicate that captopril does not

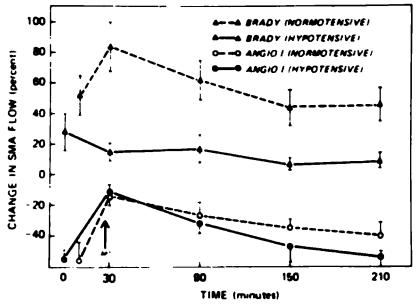

FIG. 3. Superior mesenteric artery flow (SMAF) responses to angiotensin I and bradykinin (1 μg/kg) in five normotensive and five hypotensive cats. Angiotensin I responses were significantly reduced by the CEI, however, bradykinin responses were not significantly increased under either condition. Values are means ± SEM. The arrow at 30 min indicates time of captopril (CAPT) (0.5 mg/kg) administration.

potentiate vascular effects of bradykinin in hypotensive animals. The fact that captopril only modestly potentiated the splanchnic dilator response to bradykinin, while it significantly inhibited the constrictor response to angiotensin I, suggests that inhibition of the conversion to angiotensin II may be a more fundamental action of captopril than is potentiation of bradykinin, particularly in hypotensive states.

Discussion. Angiotensin II, at circulating concentrations approximating those *in vivo* during hemorrhagic shock, prevented the prolongation of survival produced by captopril in hemorrhaged cats. This reduction in survival time may be related to diminished SMAF following the reinfusion of shed blood, since preservation of splanchnic flow has been associated with improved survival after hemorrhage (5). Thus, blockade of angiotensin II formation is probably the major beneficial action of the CEI in shock. In contrast, potentiation of the vascular effects of bradykinin does not appear to be involved in the beneficial effect of CEI in hemorrhagic shock. Since captopril did not increase bradykinin effects in hypotensive animals, this action of the CEI cannot account for the protective effect of CEI in hemorrhagic shock.

Converting enzyme inhibitors, therefore, appear to modulate the severity of hemorrhagic shock by reducing the formation of angiotensin II. Bradykinin-potentiating actions are absent at low pressures, and, therefore, are not essential to the protective mechanism of captopril. The importance of angiotensin II elimination to the beneficial effects of CEI in hemorrhage is in agreement with other studies reporting improved survival in hemorrhagic shock in animals treated with saralasin, an angiotensin II receptor antagonist (6). Also, infusion of angiotensin II has been shown to be associated with cardiac lesions and deterioration of cardiac performance (7, 8). Indeed prolonged elevation of circulating angiotensin II concentrations can induce myocardial infarction (7). Thus, there is a rational basis for preventing elevated angiotensin II concentrations in hemorrhagic shock.

Summary. Anesthetized cats subjected to hemorrhage were treated with captopril, a CEI (0.5 μg/kg/hr) alone or in conjunction with angiotensin II (10 μg/kg/hr). Angiotensin II administration significantly reduced survival time (110 vs 64 min) and lowered superior mesenteric artery flow (SMAF) immediately after reinfusion (8.5 vs 2.2 ml/kg/min). These results suggest that blockade of angiotensin II formation is a prominent aspect of the beneficial actions of captopril in hemorrhage. Furthermore, captopril potentiated the vascular effects of bradykinin at normal blood pressures but not in hemorrhagic hypotension. In the same experiments, captopril blocked the vascular effects of angiotensin I at both pressures. Thus, bradykinin potentiation is not essential for the beneficial actions of captopril in hemorrhagic shock, since hypotension abolishes the bradykinin-potentiating effects of captopril. Reduction of angiotensin II formation appears to be a major mechanism of captopril action in prolonging survival after hemorrhage.

We wish to thank Dr. Michael Antonaccio of the Squibb Institute for Medical Research, Princeton, New Jersey 08540 for the generous supply of SQ-14,225 (Captopril®, Squibb).

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Persistence of Coitus during Induced Infertility in Male Rats (40618)

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During routine safety evaluation of a maltase inhibitor, 2,2-dimethyl-1-(4-methylphenyl)-1-propanone, histological preparations of testes from treated rats showed suppression of spermatogenesis. Based on histological information the alterations in sperm cell development were found to be reversible since spermatogenesis returned upon drug withdrawal. The objective of this study was to determine if the histological observations were correlated with effects on coitus and/or fertility.

Materials and methods. Adult male and nonparous female rats of Wistar origin (Royal Hart Farms) were used in the breeding study. All animals were housed individually in open wire-bottom cages in a room with a 14:10 light/dark photoperiod. Midpoint of the light period was 12:00 noon.

The breeding study spanned a 27-week period as outlined in Table I. Nonparous females were exposed only to the pelleted ration. During cohabitation the medicated ration was withdrawn overnight from the male cage and returned the following morning when the female was returned to her cage. Cohabitation was allowed on Monday, Tuesday, or Wednesday of each week and males were exposed individually to proestrus females until a mating occurred. Copulation was verified by the presence of sperm cells in the vaginal smears and/or copulation plugs. If a male failed to copulate on all 3 days, he was not exposed to another female until the following week.

Male fertility was assessed by the presence or absence of fetuses in females autopsied 19–21 days postcoitus for the first 20 weeks of the study. The last 7 weeks it was based upon actual littering data.

The photographs of the histological preparations used in this paper were obtained in part from the safety evaluation study referred to in the introduction. Adult Sprague–Dawley males (Charles River Farms) were housed individually in open wire-bottom cages and fed the drug in the diet for 8 weeks as in the current study. The concentration of the drug in the diet (see below) was identical for the two studies. Tissue samples were obtained from Sprague–Dawley males fed the drug for 2, 4, or 8 weeks as well as from males autopsied 4 weeks following drug withdrawal. Wistar males in the current study were used to provide tissue samples from males autopsied 8 weeks following drug withdrawal. Tissues from both studies were fixed in Bouins solution and 5-μm sections stained with hematoxylin–eosin for microscopic evaluation.

The compound, 2,2-dimethyl-1-(4-methylphenyl)-1-propanone, was initially sprayed onto a stock of ground rat chow by dissolving it in acetone. Following evaporation of the acetone the stock was mixed using a Hobart feed mixer with the entire quantity of ration to be fed. The drug was incorporated at a concentration that would expose each male to an equivalent of 100 mg/kg (0.1% of the diet).

Results. A graphic representation of the results obtained for the parameters investigated is presented in Figs. 1A–D. Males in both groups were found to be fertile during the 8-week pretreatment period.

The various parameters remained unchanged among control males during the treatment period (Weeks 9 through 16). However, among treated males the number of females containing sperm cells in the vaginal smears fell throughout the treatment period (Fig. 1B). By the fourth week the number of sperm cells were greatly reduced and none were normal in appearance. Sperm cells were completely absent from the vaginal smears of females exposed to treated males during the last week (Week 16) of exposure to the drug. All treated males continued to copulate, since copulation plugs were observed under the male cages.

Litter sizes recorded for females exposed to

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TABLE I. EXPERIMENTAL DESIGN FOR BREEDING STUDY

Type of ration administered[a]	Week(s) of study	Number of Males	Number of Weeks fed	Observations
Pelleted	1–5	26	5	Sperm cells, vaginal plugs, number fetuses
Ground	6–8	26	3	Sperm cells, vaginal plugs, number fetuses
Ground	9–16	26	8	Sperm cells, vaginal plugs, number fetuses
(a) Nontreated		13		
(b) Treated[b]		13		
Pelleted	17–27	26	11	Sperm cells, vaginal plugs, litter size

[a] Purina Rat Chow.

[b] 2,2-Dimethyl-1-(4-methylphenyl)-1-propanone, concentration = 0.1% of diet.

treated males were similar in size to controls after 6-day exposure to the drug (Fig. 1D). Only one female cohabitated with treated males was found to be pregnant at autopsy during the second and third week of drug treatment. Both pregnancies were attributed to different males. Though all five fetuses were observed to be undergoing resorption at autopsy for the female sacrificed during the second week of drug exposure, all 16 fetuses were found to be normal in the female autopsied during the third week of drug exposure.

Microscopic examination of the testes at the second week of drug treatment revealed disruption of Sertoli cells and presence of syncytial giant cells in the lumen of some seminiferous tubules compared to the findings in controls (Figs. 2A and C). The presence of giant cells and cellular debris as well as a noticeable reduction in the number of sperm cells was noted in the epididymis of treated males (Figs. 2B and D). The seminiferous tubules of males autopsied on the fourth and eighth week of drug treatment were completely free of the latter stages of germ cell maturation and some contained giant cells (Figs. 3A and C). The tubules of the epididymis were free of spermatozoa and occasionally contained giant cells along with cellular debris during this time (Figs. 3B and D).

Sperm cells were noted in the vaginal smears of females exposed to control males throughout the study (Fig. 1B). Among females exposed to treated males the vaginal smears showed numerous sperm cells during the pretreatment period (Fig. 1B). However, during the treatment period the number of females with sperm cells in the vaginal smears as well as the number of sperm cells present declined throughout this period, even though treated males were copulating as evidenced by the presence of copulation plugs under each male's cage. Following withdrawal of the compound sperm cells did not reappear, with but one exception, until the fifth week and in only 2 females (neither pregnant) at this time (Figs. 1B and C). Six weeks after discontinuation of treatment 9 females were inseminated (Fig. 1B); 4 delivered litters slightly smaller in size than controls (Figs. 1C and D). Thereafter 9 to 10 females cohabitated with treated males were inseminated each week and the majority delivered litters of normal size (Figs. 1B, C, and D).

Microscopic examination of tissue taken 4 weeks following drug withdrawal revealed that the vast majority of seminiferous tubules had returned to normal (Fig. 4A). However, occasional seminiferous tubules not fully recovered were observed. Tubules of the epididymis were devoid of spermatozoa and contained cellular debris (Fig. 4B). Both seminiferous tubules and epididymides were found to be normal in all but one of the treated males 8 weeks following drug withdrawal from the diet (Figs. 4C and D).

Discussion. Abnormal levels of glucose in the testes of rats impairs spermatogenesis as a result of degenerative changes in the germinal epithelium (1). The importance of glucose in maintaining normal morphology of the testes is illustrated by the observations of Mancini *et al.* (2) who reported several types of alterations in the germinal epithelium of rats in which hypoglycemia had been induced experimentally. Zysk *et al.* (3) recently reported that 5-thio-D-glucose, an inhibitor of glucose active transport, fed in the diet of mice for 3 weeks inhibited spermatogenesis. Whether its effect on testicular function was by way of its inhibitory effect on glucose

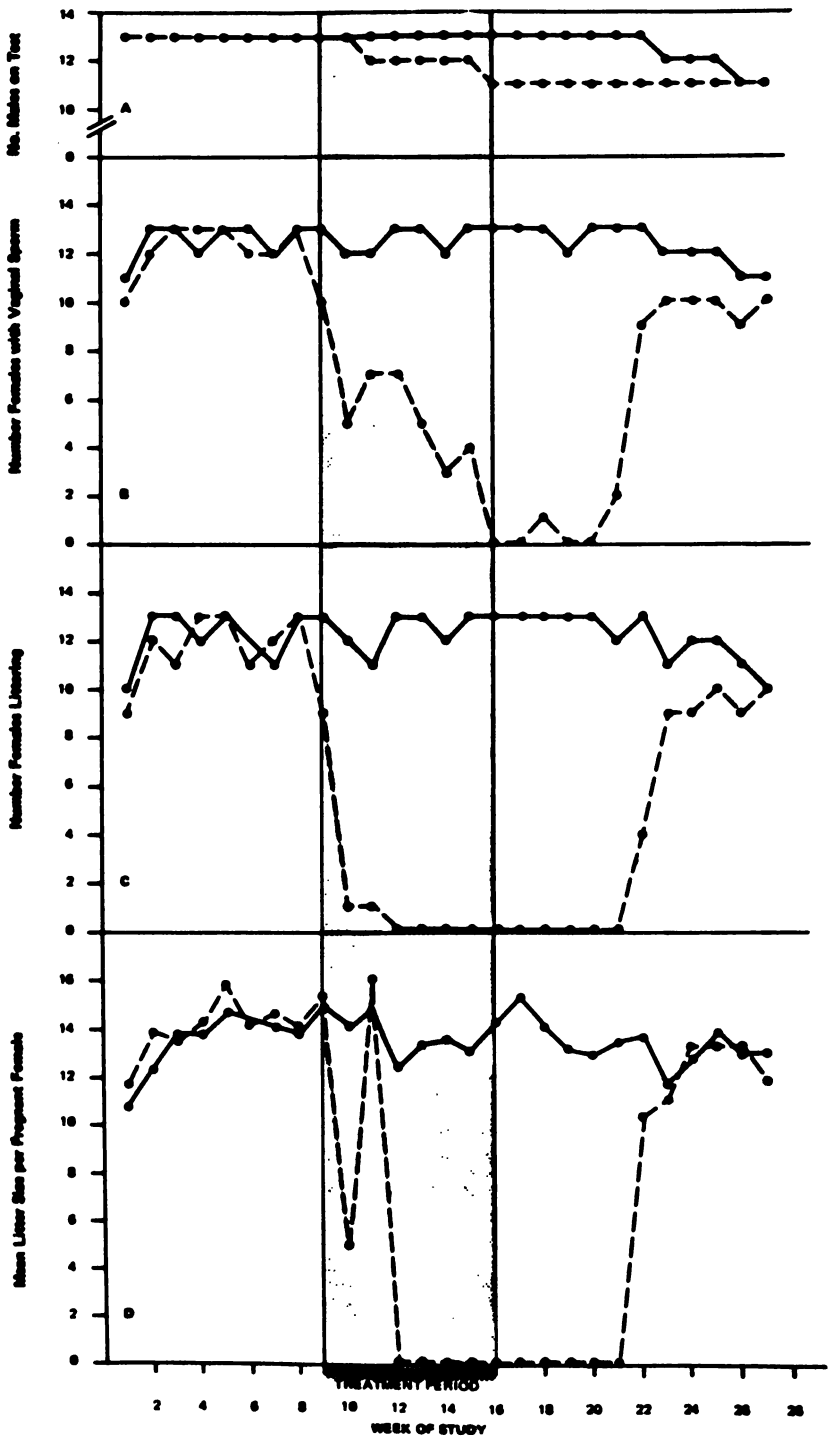
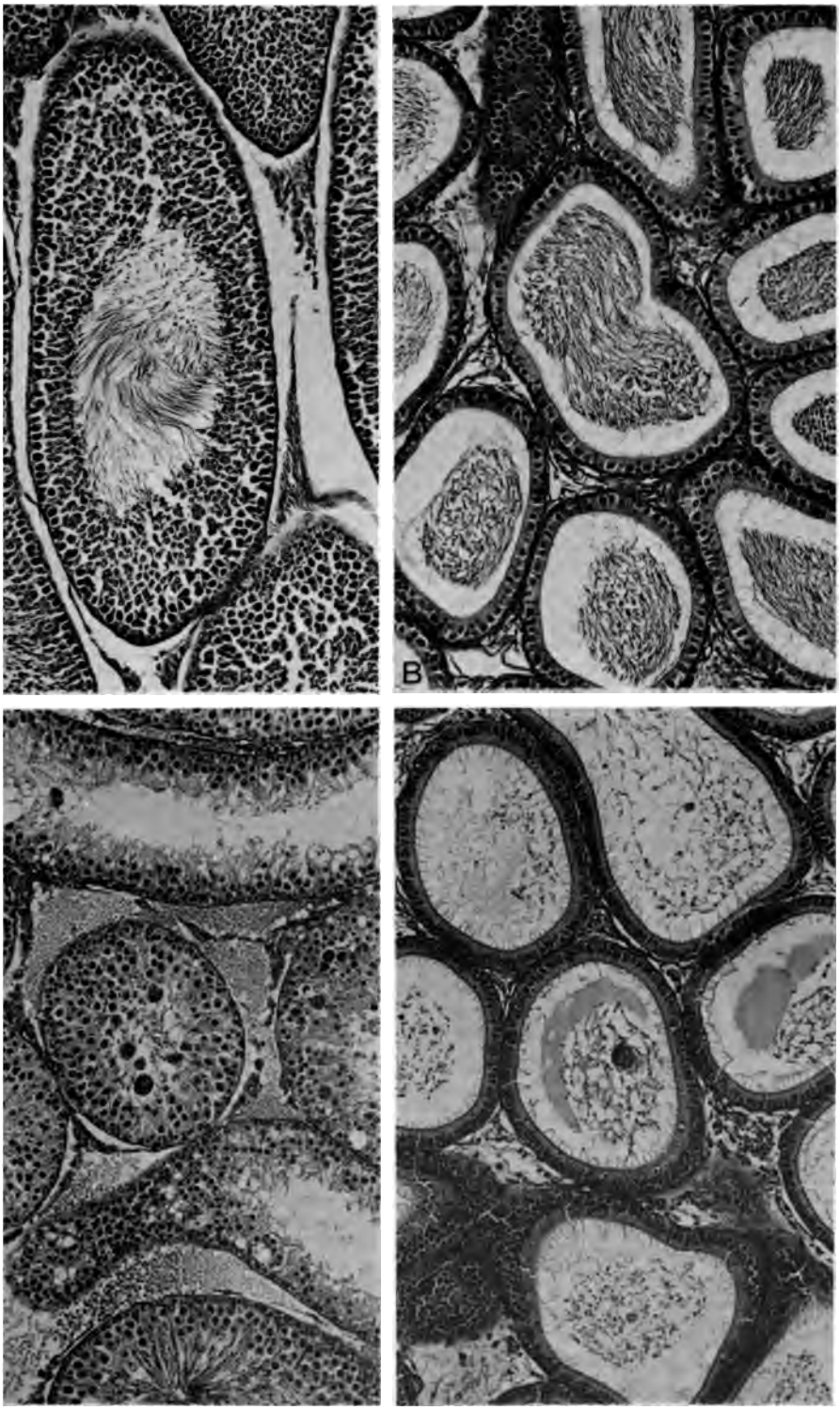


FIG. 1. The effect of 2,2-dimethyl-1-(4-methylphenyl)-1-propanone on fertility in male rats (●——● = con ·--· = treated).



miniferous tubules (A) and epididymis (B) obtained from a control male. Interruption of normal germ
n and presence of syncitial giant cells are seen (C) in the seminiferous tubules of a rat treated for 2
idydimis (D) of the same rat contained both giant cells and cellular debris. Note in particular the lack
a (× 332.5).

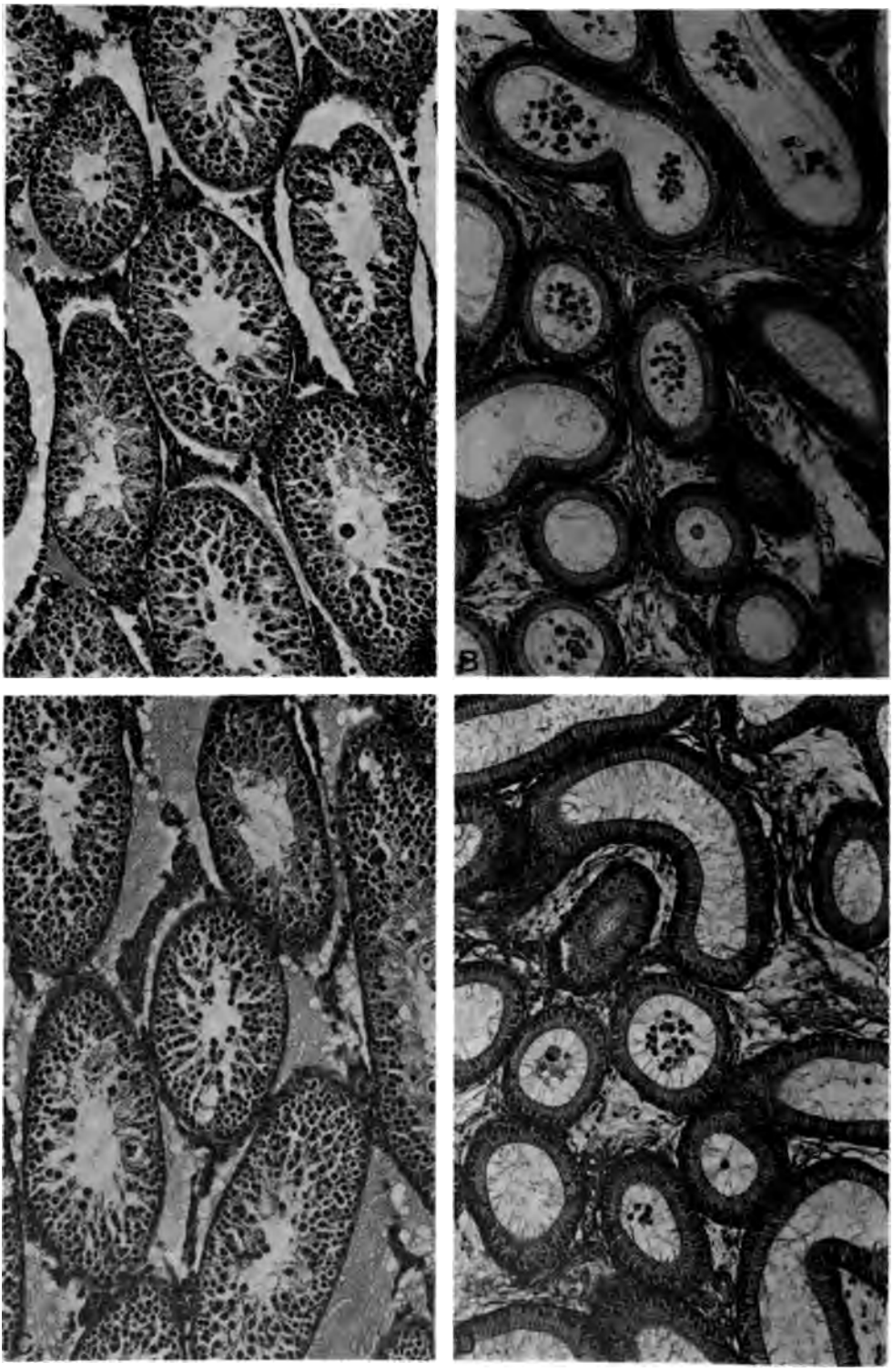


FIG. 3. Seminiferous tubules (A) of a male after 4 weeks of treatment contained reduced numbers of spermatogonia and primary spermatocytes and are completely devoid of spermatids. The epididymis (B) contained only scanty cellular debris. The seminiferous tubules (C) and epididymis (D) of males treated for 8 weeks were similar to those treated for 4 weeks (× 332.5).

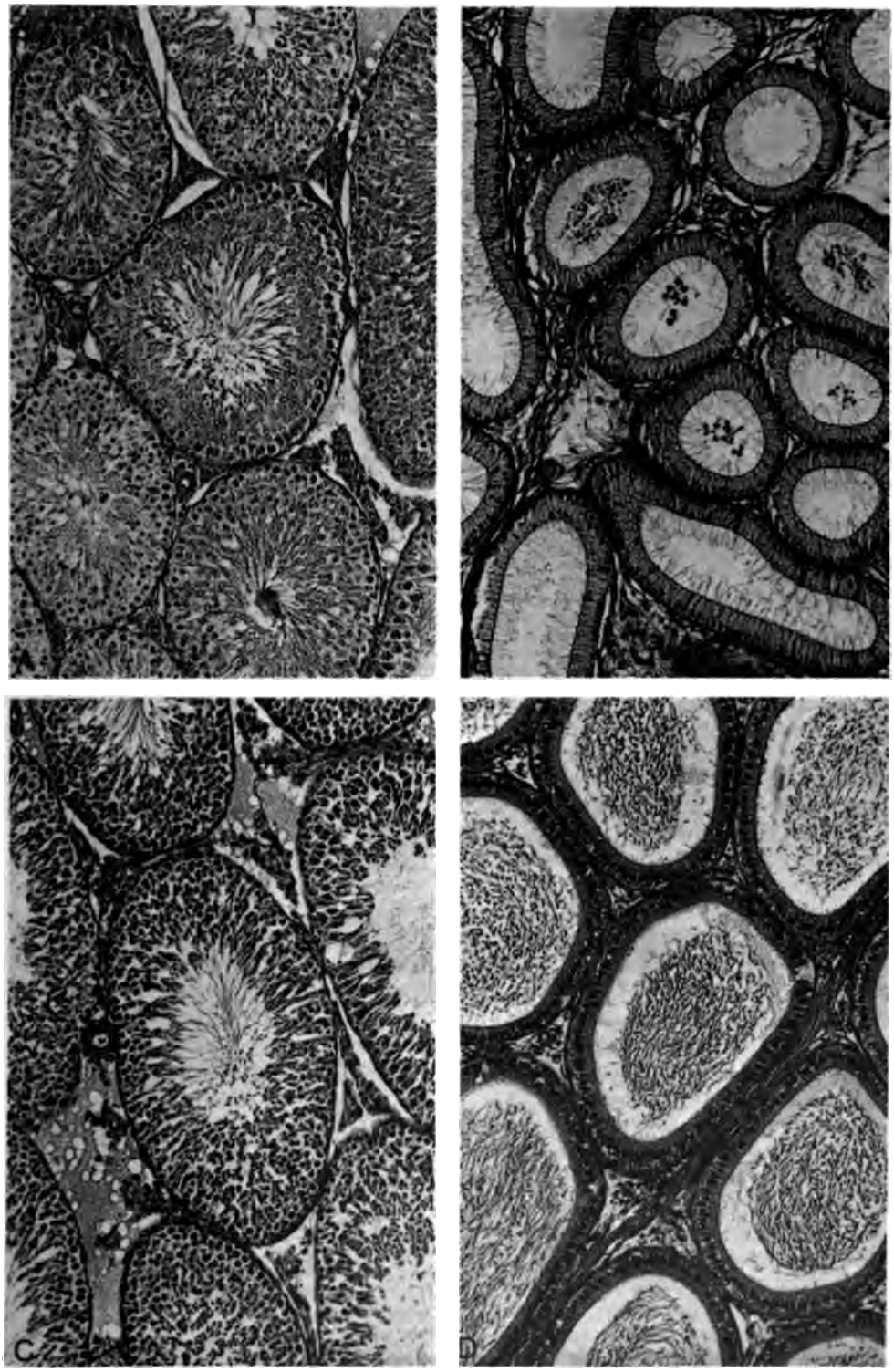


FIG. 4. Four weeks following drug withdrawal the seminiferous tubules (A) showed normal germ cell maturation d presence of spermatids. The epididymis (B) was devoid of spermatozoa and contained only cellular debris. The niniferous tubules (C) and epididymis (D) were found to be completely normal 8 weeks following drug withdrawal 332.5).

active transport across cell membranes or by some unknown mechanism of action was not determined.

It is clear from this study that the testicular changes observed with the administration of a maltase inhibitor were reversible upon withdrawal of the compound from the diet. Evidence for this was based not only upon microscopic examination of the testes of treated males, but also upon resumption of fertility in breeding studies.

Equally important was the observation that treated males continued to copulate during the period of time males were found to be infertile. Sperm cells or fragments thereof were noted in the vaginal smears of females throughout 7 of the 8 weeks of drug treatment. During the eighth week of exposure to the drug, copulation plugs were recorded under the cage of each treated male even though sperm cells were completely absent from the vaginal smears at this time. In addition copulation plugs continued to be observed under each male's cage during the period of time following drug withdrawal that the males remained infertile.

The testicular changes obtained in this study bear a striking resemblance to those reported in rats in which hypoglycemic comas were induced with insulin (2) as well as mice fed 5-thio-D-glucose (3). Since both studies (2, 3) reported similar types of alterations to those found in this study, it may be the changes were associated with a testicular deficiency in available glucose.

Mancini *et al.* (2) concluded that in their study the testicular changes were not readily reversible since they observed incomplete recovery of testicular damage 5 weeks following induction of the last hypoglycemic coma. In this study the alterations were detectable in some seminiferous tubules of a few males 4 weeks following drug withdrawal but germ cell development was proceeding normally in the vast majority of seminiferous tubules, and by the eighth week testes were found to be completely normal histologically. Thus, one may conclude that the differences between these two studies may reflect in part the type of agent used to inhibit spermatogenesis as well as time of autopsy.

Zysk *et al.* (3) found that administration of 5-thio-D-glucose in the diet of male mice inhibited spermatogenesis within 3 weeks and remained so for 7 weeks without impairment of libido. Resumption of normal fertility was obtained within 5 to 8 weeks following removal of the compound from the diet. A similar pattern was observed in this study insofar as fertility was completely inhibited by the fourth week of drug treatment and remained so for 5 weeks following drug withdrawal from the diet without preventing coitus.

Clinically a compound which would inhibit spermatogenesis during administration without compromising libido and reverse its effect on spermatogenesis upon withdrawal within a reasonable length of time would be highly desirable as a male contraceptive. In this regard a nonsteroidal agent would be more desirable than a steroidal agent with its attendant hazard of disruption of the normal hormonal milieu. The findings obtained in this study as well as those reported by Zysk *et al.* (3) demonstrate in rodents that such a concept is certainly valid. Indeed, 2,2-dimethyl-1-(4-methylphenyl)-1-propanone may represent a chemical lead in the development of a clinically effective male contraceptive.

Summary. Incorporation of 2,2-dimethyl-1-(4-methylphenyl)-1-propanone into the diet of male rats inhibited fertility within 4 weeks. Examination of the testes showed that spermatogenesis was inhibited and they remained infertile without preventing coitus throughout the treatment period. Six weeks following withdrawal of the compound from the diet, litters of normal size were sired by treated males. The results obtained in this study demonstrate that male fertility can be inhibited without preventing coitus and that normal fertility can be realized upon drug withdrawal.

The authors wish to express their gratitude to Dr. Richard Elton for reviewing the manuscript and to Miss Cherle Knott for her secretarial assistance.

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OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 65–70 (1979)

ıethacin Blockade of Albuterol-Induced Erythropoietin Production in Isolated Perfused Dog Kidneys (40619)[1]

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ırmation and/or release of erythro-
Ep) by the kidney have recently been
ated to be under the control of both
l (1–4) and neural (3, 5, 6) influ-
ıus, Ep production following expo-
ypoxia has been found to be abol-
cholinergic (6) and β-adrenergic (3)
or renal denervation (3). In addi-
laboratory has previously reported
vation of the β-adrenergic nervous
ıcreases Ep production in normoxic
(7). Albuterol, a specific β-2 adre-
onist (8), has been found to produce
ant increase in serum Ep titers when
ered to rabbits (7, 9). This effect was
l by the simultaneous administration
ıdrenergic blocking agent (9). Thus,
clear from these *in vivo* studies that
t of albuterol on Ep production is
by β-2 adrenergic receptors. On the
nd, the role of prostaglandins inhi-
the direct effects of β-2 adrenergic
on Ep elaboration in renal cells has
ofore been investigated.

ore, we have undertaken studies of
ts of albuterol on Ep production in
ned isolated posthypoxic dog kid-
ng normoxemic and hypoxemic per-
ı addition, experiments were carried
termine if the postulated β-2 adre-
tivation of Ep production requires
l synthesis of renal prostaglandins,
en shown for the elaboration of Ep
ypoxia (2), by studying the effects of
ıacin, an inhibitor of prostaglandin
, on albuterol-induced kidney pro-
of Ep.

als and methods. Female mongrel
-25 kg, b.w.) with hematocrits be-
tween 35–44% were exposed to hypoxia (0.42 atm) for 2 hr prior to removal of the kidney for perfusion. The left kidney (18–58 g) was removed under sodium pentobarbital (30 mg/kg i.v.) anesthesia via abdominal laparotomy and renal arteries and ureter cannulated with polyethylene tubing. At the same time 500 ml heparinized blood (500 U sodium heparin/kg b.w.) was collected from the carotid artery to be used as the perfusate. Kidneys were flushed with a single pass of 500 ml heparinized (0.5 U/ml) lactated Ringer's solution at 37° to remove the blood contained in the kidney. Thereafter, the kidneys were placed in a closed-circuit perfusion system consisting of an organ-warming chamber, membrane blood oxygenator, blood reservoir, heating coil, and perfusion pump as described previously (10). Isolated kidneys were perfused for 5 hr at 37° at a flow rate of 3 ml/g/min with 500 ml recirculating blood (adjusted to a hematocrit of 35%). Urine was returned to the perfusion system. Blood gases were analyzed (Instrumentation Labs Model 127 blood gas analyzer) initially and at hourly intervals. Blood pO_2 and pCO_2 were kept constant by equilibration with N_2, CO_2, and O_2 in a sialastic tubing oxygenator (arterial pCO_2 35–45 mmHg, pH 7.35–7.45). Arterial pO_2 was maintained between 60–80 mm Hg in normoxemic perfusions and between 18–25 mm Hg in hypoxemic perfusions. Perfusion pressures (Statham pressure transducer P23AC) and perfusate temperature were monitored continuously. The following substances were regularly added to the perfusion fluid: 500,000 U of penicillin G at the beginning of the experiment and subsequently 100,000 U at 2 and 4 hr of perfusion; 2000 U sodium heparin at 2 and 4 hr; and 75 mg glucose hourly. Albuterol (500 μg/liter blood, Schering Inc., N.J.) dissolved in saline or saline alone (50 μl) was added to the blood prior to the perfusion. Blood samples (20 ml) for the determination of plasma erythropoi-

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etin titers were removed from the perfusate after 5 min (0-hr value), 3 and 5 hr of perfusion.

In the experiments where indomethacin was used the dogs were pretreated with the drug (5 mg/kg p.o.) 16 hr and 15 min before exposure to hypoxia. The erythropoietic activity of the plasma samples from the perfusion system was determined using the exhypoxic polycythemic mouse bioassay for Ep (11). HAM/JCR strain female mice were made polycythemic by exposure to hypoxia for 2 weeks (0.42 atm for 22 hr/day). Groups of five mice were injected s.c. with either saline, human urinary Ep standardized against the International Reference Preparation, or 0.5 ml plasma on Days 6 and 7 after their removal from the hypobaric chamber. On Day 8 the animals received 0.5 μCi ^{59}Fe citrate i.v. Blood was removed via cardiac puncture on Day 10 and erythropoietic activity of the sample was calculated from the mean 48-hr percentage ^{59}Fe incorporation into red blood cells for each of the mice. Experiments in which the initial erythropoietic activity (0-hr value) of perfusate samples exceeded 5% ^{59}Fe incorporation in the mouse bioassay were excluded from the results (4 out of 34 experiments).

In order to prove that the erythropoietic activity of perfusate samples was due to erythropoietin and not to possible effects of the drug remaining in the plasma samples being assayed on bone marrow progenitor cells (9), the effect of antierythropoietin immune serum (anti-Ep) on the erythropoietic activity of plasma samples was assessed. Plasma was incubated with rabbit anti-Ep serum or normal rabbit serum for 45 min at 37°, followed by removal of the anti-Ep by an additional incubation with goat anti-rabbit γ-globulin (GARGG) for 45 min at 37°. The plasma was then centrifuged and the supernatant assayed for erythropoietin in the polycythemic mouse bioassay. The Ep assay results are expressed as the mean ± SEM percentage ^{59}Fe incorporation in red cell values. The statistical significance of the data was calculated using Dunnett's test for multiple comparisons with a single control or the Student's *t* test for single comparisons.

Results. Effect of albuterol during normoxemic perfusion. As noted in Fig. 1, no significant erythropoietic activity was found in saline-control perfusates at the beginning of the perfusion period (0-hr value). In addition, no increase in Ep titers was observed during a 5-hr perfusion of the isolated programmed posthypoxic dog kidneys with normoxemic (pO2 60–80 mm Hg) blood. When albuterol (500 μg/liter) was added to the blood prior to the beginning of the perfusion, a significant increase in Ep titers was seen in the perfusates. Plasma Ep titers were significantly (*P*

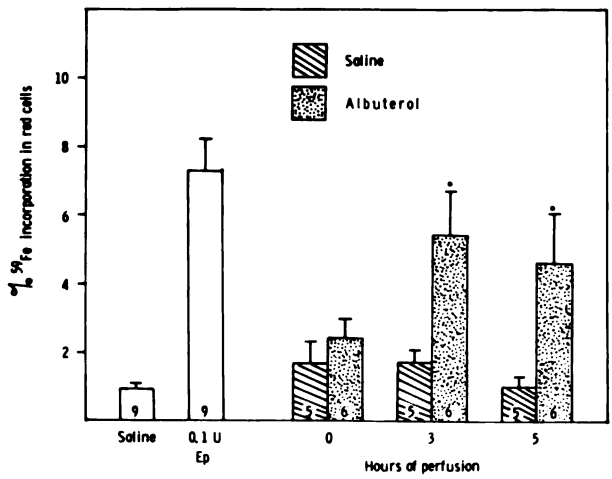


FIG. 1. Effects of albuterol (500 μg/liter) on Ep production in the normoxemic (pO_2 60–80 mm Hg) isolated perfused dog kidney. Each bar is the mean ± SE (numbers at the bottom represent number of experiments). *Significantly ($P < 0.05$) different from saline control value.

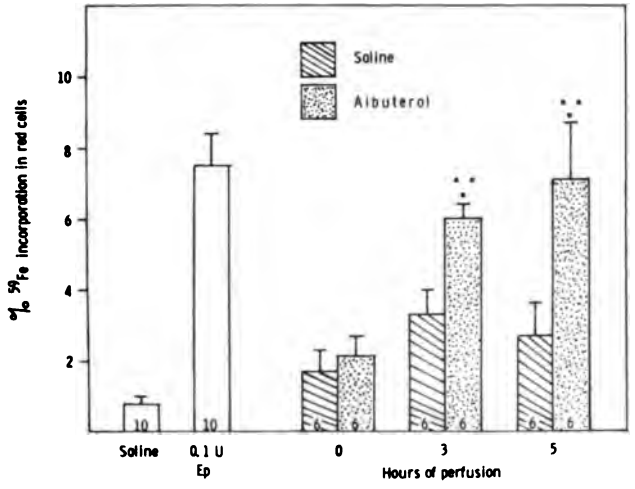
gher in albuterol normoxemic than
ıormoxemic perfused kidney per-
er 3 and 5 hr.

f albuterol on erythropoietin produc-
g hypoxemic perfusion. The eryth-
activity in plasma perfusates from
ıoxemic (pO_2 18–25 mm Hg) per-
; kidneys after 3 hr of perfusion
72 percentage ^{59}Fe incorporation in
; significantly ($P < 0.05$) increased
level detectable in the polycythemic
ɔassay (0.82 ± 0.14%). As demon-
Fig. 2, Ep titers were moderately
above their respective 0-hr value
d 5 hr saline-hypoxemic perfusion
creases were not statistically signif-
the other hand, when albuterol was
the perfusate a significant increase
rs was found at 3 and 5 hr when
with that of either saline-hypox-
ɪsate or with the initial 0-hr value.
ssibility that the albuterol in the
and not Ep was responsible for the
ietic stimulation in the polycy-
ce was ruled out by incubating the
erol perfusates with either anti-Ep
ı or normal rabbit serum. After the
was removed by GARGG, the
ietic activity was abolished in the
eated perfusate samples, whereas
ı with normal rabbit serum did not
titers when assayed in exhypoxic
ɪic mice. These results indicate
that the plasma perfusates contained erythropoietin.

Effect of indomethacin pretreatment on albuterol-induced Ep production. As shown in Fig. 3 when dogs were pretreated with indomethacin prior to exposure to hypoxia, no detectable Ep was found during a 5-hr albuterol-normoxemic perfusion. Indomethacin pretreatment caused a decrease in Ep production which was significantly below that seen in nontreated dog kidneys following the addition of albuterol to the perfusate.

Perfusion pressure. In maintaining a constant renal blood flow (3 ml/g/min) during the perfusion some changes occurred in the perfusion pressure. Following an initial autoregulatory decrease in perfusion pressure, a gradual increase in pressure was usually seen over the 5-hr perfusion period. However, no significant differences were found between the treatment groups and Ep production was not correlated with the perfusion pressure in any of our experiments. Mean perfusion pressures at 30 min and 5 hr were 114 and 148 mm Hg without pretreatment of dogs and 119 and 191 mm Hg, respectively, following indomethacin pretreatment.

Discussion. We have previously reported that β-2 adrenergic agonists increase plasma Ep titers in normoxic (7, 9) and hypoxic (12) animals. The present study was undertaken to determine whether this effect is due to a direct renal cell adrenergic receptor activa-



Effects of albuterol on Ep production in the hypoxemic (pO_2 18–25 mm Hg) isolated perfused dog kidney. the mean ± SE (numbers at the bottom represent number of experiments). *Significantly ($P < 0.05$) n saline control value. **Significantly ($P < 0.05$) different from the respective 0-hr value.

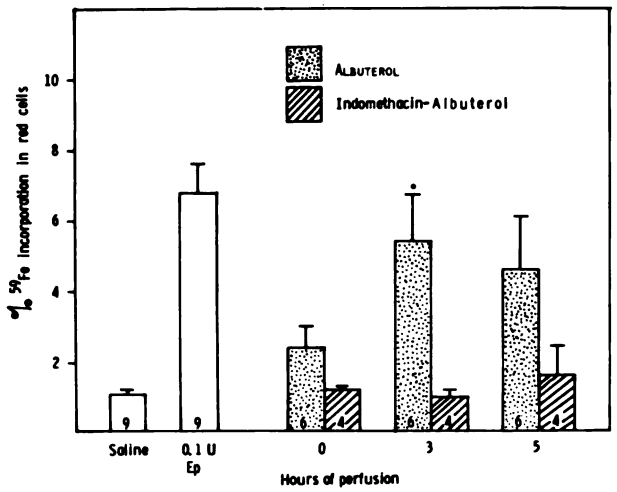


FIG. 3. Effects of pretreatment with indomethacin (5 mg/kg twice) on albuterol-induced Ep production in normoxemic isolated perfused dog kidneys. *Significantly ($P < 0.05$) different from indomethacin pretreatment.

tion of Ep production or to indirect effects due to a β-2 adrenergic activation of prostaglandins release in the kidney.

β-Adrenergic agonists are known to influence respiratory ventilation, blood pressure (8), and renal blood flow (13). Isolated dog kidneys were perfused at a constant flow rate in the present experiments in that the production of Ep is known to be modified by changes in renal blood flow (14). On the other hand, we were unable to detect a correlation between the perfusion pressure and Ep elaboration. In contrast to our earlier studies (10) the current perfusion technique utilized the blood and kidneys from the same dog in the perfusion system. No detectable Ep was found in the blood removed from the 2-hr posthypoxic dogs which was the 0-hr perfusate sample. In addition, no Ep was released from kidneys during the 5-hr perfusion with normoxemic blood [>90% oxy-Hb (15)]. During hypoxemic perfusion ($\triangleq$ 20% oxy-Hb), erythropoietic activity was significantly higher at 3 hr than the ^{59}Fe incorporation values seen in saline-treated polycythemic mice. Addition of albuterol to the perfusate significantly enhanced Ep production in normoxemic and hypoxemic perfused kidneys when compared to saline-control kidney perfusates. The possibility that the erythropoietic activity of the albuterol perfusates was not Ep but caused by direct effects of albuterol on the mouse bone marrow erythroid progenitor cells (9) was ruled out since Ep titers were lower at 0 hr when compared to that of the later time intervals. In addition, erythropoietic activity in the plasma perfusate was also abolished following incubation with anti-Ep.

The significance of β-adrenergic activation in the physiologic control of Ep production is not clear at the present time. Ep is certainly produced at an accelerated rate in adaptation to hypoxia (1). The administration of the β-adrenergic antagonist propranolol has been shown to partially antagonize Ep elaboration during hypoxia (3). In addition, catecholamine levels are known to be increased during hypoxia (16). The concentration of catecholamines in blood of normal dogs is estimated to be 10^{-8} M which is lower than that of the initial concentration of albuterol (10^{-6} M) used in our experiments. However, it is likely that a major part of the β-2 adrenergic agonist is rapidly bound to cell surfaces so that the actual concentration of the free drug at the appropriate receptor site is much lower than can be calculated from the initial concentration. It should be noted that in general marked pharmacological stimulation of Ep release is necessary to produce titers of Ep that can be detected with the biological assay methods currently available which is at best greater than 0.05 U Ep/ml. Considering these data, our results support the hypothesis that the β-adrenergic nervous system plays a physiological role in the regulation of Ep production during adaptation to hypoxia.

It is of interest to consider the possible

nisms by which β-2 adrenergic activa-
ıcreases Ep production in the kidney.
lrenergic agonists in general have been
ıstrated previously to exert their hor-
effects by increasing cell membrane
late cyclase activity and thereby result
increase in the intracellular concentra-
f cAMP (17). β-Adrenergic agonists are
:apable of increasing renal cortical
levels *in vitro* (18). Our earlier work
ıggested that the synthesis of Ep is
ted by an increase in renal cAMP levels
Therefore, it seems very likely that al-
ıl increased Ep production by the acti-
of adenylate cyclase to increase cAMP
ıtrations in a renocortical cell in the
:d perfused kidney either directly or
;h prostaglandins release.

:onsidering the current finding that in-
hacin blocked the effects of albuterol
lney Ep production, evidence has been
led from our laboratory that renal pros-
dins, primarily PGE_2, are involved in
echanism of kidney elaboration of Ep
; hypoxia (2, 10). When dogs were pre-
l with the prostaglandin synthetase in-
r indomethacin prior to exposure to
ia, Ep production was abolished during
ic stimulation (2). The results presented
ıdicate that β-2 adrenergic activation
production may also be mediated by a
glandin mechanism since indometha-
ıcked the enhanced Ep production with
rol. However, one must consider the
ility that pretreatment of the dogs with
ethacin may also have inhibited the
!-hr programming phase of the kidney
arly stage of the Ep cascade. Enhanced
: of prostaglandins from the kidney has
eported to occur immediately after the
ing of hypoxia (20, 21). We have pos-
l previously that limited exposure to
ıa renders the kidney more responsive
hropoietic stimuli (10), especially since
/el of the inactive form of the renal
)poietic factor (REF, erythrogenin) in
ney has been shown to increase during
a (19). It is quite possible that indo-
:in pretreatment prior to the 2-hr ex-
of the dog to hypoxia blocked the
sis of prostaglandins during the pro-
ing phase and prevented the elabora-
REF (erythrogenin) and thereby de-
creased the effects of albuterol on an early phase of albuterol's effect on Ep production in the isolated perfused kidney. It would be of interest to learn in further studies in the future whether a prostaglandins synthetase inhibitor added directly to the perfusate would interfere with albuterol-induced kidney production of Ep. Our current results do strongly suggest that albuterol exerts its effects, at least in part, by increasing the synthesis of a renal prostaglandin which plays an important role in kidney production and/or release of Ep. Even though β-adrenergic agonists have been implicated in the release of prostaglandins from the kidney (22), further studies on the effects of β-2 adrenergic agonist activation of prostaglandins synthesis and Ep production are necessary to establish the role of renal prostaglandins in β-adrenergic activation of kidney production of Ep.

Summary Albuterol, a specific β-2 adrenergic agonist, was found to produce a significant increase in perfusate levels of Ep in isolated perfused dog kidneys which were programmed by prior exposure to hypoxia (0.42 atm) for 2 hr. Albuterol enhanced Ep titers in perfusates in both normoxemic and hypoxemic perfused kidneys which were significantly higher than that of the Ep level seen in saline controls during a 5-hr perfusion period. The effect of albuterol on Ep production in isolated perfused kidneys was abolished by pretreatment of the dogs with the prostaglandins synthetase inhibitor indomethacin. The inhibition of albuterol-induced Ep elaboration by indomethacin supports our hypothesis that prostaglandins may play a significant role in mediating β-2 adrenergic activation of kidney Ep production.

The expert technical assistance of Mr. Eugene Maulet is gratefully acknowledged.

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nal and Hepatic Mixed Function Oxidase Activity in Rats Fed Methionine and Cysteine-Free Diets (40620)

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opic agents function in methyl group or are methyl donors during the syn- phospholipids, which are essential ceride transport from the liver. Rats marginally deficient in lipotropic ow a greater incidence of tumors in to several different carcinogens (1– e above cited experiments the diets in the lipotropic agents choline, ne, and folic acid, deficient in niacin, her indispensible amino acids, and fat. Supplementation of these diets tropic agents or a mixture of indis- amino acids decreased the number ylnitrosamine (DEN)-induced he- ors (5). Methionine, which was con- both the amino acid and lipotrope nts may have contributed to re- mor incidence. In another study latoxin B_1-induced hepatic tumors nd in rats fed a basal diet containing protein supplemented with choline, ne, and vitamin B_{12}. Soybean pro- st limiting in methionine (6).

on has major influence on hepatic mixed function oxidase (MFO) en- nd may thereby alter cancer inci- *vitro* hepatic MFO activity is re- rats fed lipotrope-deficient diets (7, studies show that the clearance of m the blood is depressed in rats fed -deficient diets (9). Pentabarbital-in- eping time is prolonged in rats fed -deficient diets (10). The above stud- st that the ability to metabolize and ne chemicals was decreased.

ort herein investigations with diets g only L-amino acids as sources of itrogen to determine if withholding ne and cysteine while keeping other factors constant would depress ivity. Described are *in vitro* hepatic l intestinal MFO activity of rats fed mino acid-free diet for 4 days. Aryl hydrocarbon hydroxylase (AHH), a complex cytochrome *P*-450 (*P*-448)-dependent MFO system which metabolizes a variety of substrates including carcinogenic polycyclic aromatic hydrocarbons was chosen as the model for study. Cytochrome b_5, the only other cytochrome in the microsomal fraction, was also examined. Cytochrome b_5 has numerous metabolic functions (11) including desaturation of fatty acids and may also be involved in some P-450-linked drug oxidations (12). Pair-feeding techniques were employed to determine the influence of the sulfur amino acid deficiency independently of reduced food consumption which occurs when one or more essential amino acids are deficient.

Materials and methods. Male, Sprague–Dawley rats averaging 138 ± 2 g in initial body weight were fed the complete L-amino acid diet of Rogers and Harper (13) containing: dextrin 49.32%, sucrose 25.68%, corn oil 5%, Rogers and Harper salt mixture 5% (Teklad Test Diets, Madison, Wi.),[1] vitamin fortification mixture 1% (Teklad Test Diets, Madison, Wi.),[2] and L-amino acids 14%. The

[1] The Rogers and Harper salt mixture contained (in percent): $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.0025%; $CaCO_3$, 29.29%; $CaHOP_4\cdot 2H_2O$, 0.43%; $CuSO_4$, 0.156%; $Fe(C_6H_5O_7)\cdot 6H_2O$, 0.623%; $MgSO_4\cdot 7H_2O$, 9.98%; $MnSO_4\cdot H_2O$, 0.121%; KO, 0.0005%; KH_2PO_4, 34.31%; NaCl, 25.06%; $Na_2SeO_2\cdot 5H_2O$, 0.0015%; $ZnCl_2$, 0.020%. Catalog No. 170760. Teklad Test Diets, Madison, Wis. The salt mixture used is low in zinc and deficient in certain trace minerals such as chromium and fluoride. The use of this mixture in purified diets could confound results in studies of longer duration.

[2] The vitamin mix contained (in percent of vitamin mix): *P*-aminobenzoic acid, 1.10132; ascorbic acid, coated (97.5%), 10.16604; biotin, 0.00441; vitamin B_{12} (0.1% trituration in mannitol), 0.29736; calcium pantothenate, 0.66079; choline dihydrogen citrate, 34.96916; folic acid, 0.09982; inositol, 1.10132; menadione (vitamin K_3), 0.49559; niacin, 0.99119; pyridoxine HCl, 0.22026; riboflavin, 0.22026; thiamine HCl, 0.22026; retinyl pal-

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nitrogen lost by complete deletion of methionine plus cysteine was replaced with glycine. All of the diets were suspended in agar gel and refrigerated until fed. Thirty-six rats were fed the control diet for 3 days before their random assignment to dietary treatments (12 per diet): control diet fed *ad libitum*, methionine-free diet fed *ad libitum*, control diet pair-fed to the intake of rats fed the methionine-free diet. After consuming their experimental diets for 4 days the rats were killed by decapitation and microsomes were isolated by differential centrifugation from the liver and the mucosa from the first 25 cm of the small intestine. Microsomal protein was assayed according to Gornall (14), cytochrome *P*-450 and b_5 as described by Omura and Sato (15), and AHH according to Nebert and Gelboin (16). Statistical analysis was by the F-LSD method (17).

Enzyme data are expressed as specific activity and total tissue activity per body weight since exposure to environmental chemicals is usually related to some function of body weight.

Results. Body weights, weight gains, food intake, liver weight, and weight of the 25-cm intestinal segment are shown in Table I. The dry matter intake was reduced approximately 50% on Day 1 for the methionine + cysteine-free diet and pair-fed rats and remained so compared to controls. Body weights were significantly reduced by pair feeding, but the weight loss of rats fed the methionine + cysteine-free diet was even greater. There were no significant treatment effects on liver or intestinal wet weights.

The total hepatic microsomal protein was lower in rats restricted in food intake and there was a further reduction with sulfur amino acid deficiency (Table II). The hepatic content of cytochrome *P*-450 expressed as nmole/100 mg or as total liver content per unit body weight was significantly lowered by the deficiencies of methionine and cysteine. There were no significant differences in cytochrome b_5 content. The specific activity of AHH or total activity per unit body weight was significantly lower in the deficient rats compared to either the controls or pair-fed rats.

There were no differences in microsomal protein in the 25-cm intestinal segment (Table III). The specific activity per unit body weight of AHH was significantly lowered by sulfur amino acid deficiency but not by restricted food intake.

Discussion. A low intake of sulfur amino acids associated with a lipotrope deficiency may influence biological activity of foreign chemicals via several mechanisms. Methionine via its conversion to cysteine is a precursor for glutathione which is used by man and numerous other species to form glutathione conjugates of foreign substances (18). Depletion of hepatic glutathione has been correlated with enhanced toxicity and covalent binding of toxins to DNA and protein (19). However, Campbell *et al.* has shown that hepatic glutathione concentrations are not altered by lipotrope deficiency (8). Methionine also participates in the methylation of foreign compounds via the nucleotide, *S*-adenosyl methionine. Male rats, but not females, fed lipotrope-deficient diets show a decrease in hepatic *S*-adenosyl methionine (20).

Our present data show that dietary sulfur amino acids also participate in foreign compound metabolism by influencing the activity of MFO enzymes. AHH activity was decreased in the liver and intestine of rats fed a diet devoid of cysteine and methionine for 4 days. A similar reduction in hepatic cytochrome *P*-450 was observed with no effect on cytochrome b_5. The AHH complex converts polycyclic aromatic hydrocarbons into more hydrophilic products which are more readily excreted and usually less carcinogenic. However, during this process metabolites which are more carcinogenic than the parent compound may be transiently produced. Several studies have shown that depressed hepatic AHH activity at the time of exposure to carcinogenic polycyclic aromatic hydrocarbons is associated with enhanced tumor development (21, 22). Decreased activity of microsomal oxidases may contribute to the enhanced carcinogenesis by specific chemicals in lipotrope-deficient rats. Rogers *et al.* (7) observed a trend toward lower activity of hepatic AHH and other MFO enzymes in rats fed their lipotrope-deficient diet. Our

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mitate (500,000 U/g), 0.39648; dry cholecalciferol (500,000 U/g), 0.04405; dry tocopheryl acetate (500 U/g), 2.42291; corn starch, 46.66878. Catalog No. 40060. Teklad Test Diets, Madison, Wis.

TABLE I. BODY WEIGHT, WEIGHT GAINS, FOOD INTAKE, LIVER WEIGHT, AND INTESTINAL WEIGHT OF GROWING RATS FED AN L-AMINO ACID DIET WITH OR WITHOUT SULFUR AMINO ACIDS[a]

Treatment	Food intake (g dry matter / day)	Final body weight	Weight gain (g/day)	Liver wet wt (g)	Intestinal segment wet wt (g)
Control—*ad libitum* fed	11.6 ± 0.1[x]	162 ± 1.7[x]	5.8 ± 0.2[x]	5.6 ± 0.5	1.93 ± 0.06
Control—pair fed	5.4 ± 0.1[y]	133 ± 2.2[y]	−1.5 ± 0.1[y]	6.2 ± 0.6	1.85 ± 0.03
Met + Cys-free diet	5.5 ± 0.1[y]	121 ± 1.5[z]	−4.2 ± 0.3[z]	5.8 ± 0.5	1.92 ± 0.04

[a] Data represents mean ± SEM. Data with significant differences ($P < 0.05$) show a different superscript.

TABLE II. HEPATIC MIXED-FUNCTION OXIDASE ACTIVITY IN GROWING RATS FED AN L-AMINO ACID DIET WITH OR WITHOUT SULFUR AMINO ACIDS[a]

	Control—*ad libitum*	Control—pair fed	Met + Cys free
Total microsomal protein (mg)	295 ± 28[x]	201 ± 13[y]	167 ± 12[z]
P-450			
nmole/100 mg protein	16.3 ± 4.0[x]	14.3 ± 1.2[x]	10.5 ± 0.7[y]
nmole/100 g body wt[b]	29.7 ± 6.2[x]	21.6 ± 4.0[x, y]	14.5 ± 3.4[y]
b_5			
nmole/100 mg protein	2.0 ± 0.5	2.7 ± 0.4	2.1 ± 0.2
nmole/100 g body wt[b]	3.6 ± 0.7	4.1 ± 0.6	2.9 ± 0.4
Aryl hydrocarbon hydroxylase			
Specific activity[c]	15.9 ± 2.1[x]	16.3 ± 2.4[x]	4.6 ± 1.3[y]
Activity/g body wt[d]	28.9 ± 3.9[x]	24.6 ± 3.1[x]	6.3 ± 1.8[y]

[a] Data represets mean ± SEM. Data with significant differences ($P < 0.05$) show a different superscript.
[b] nmole/100 mg protein × total microsomal protein ÷ body wt × 100.
[c] nmole 3-OH benzo(a)pyrene/30 min/mg protein.
[d] Specific activity × total microsomal protein ÷ body wt.

TABLE III. INTESTINAL MIXED-FUNCTION OXIDASE ACTIVITY IN GROWING RATS FED AN L-AMINO ACID DIET WITH OR WITHOUT SULFUR AMINO ACIDS[a]

Treatment	Control—*ad libitum*	Control—pair fed	Met-Cys deficient
Total microsomal protein, 25-cm segment (mg)	15.7 ± 0.4	14.8 ± 0.5	15.0 ± 0.4
Aryl hydrocarbon hydroxylase			
Specific activity[b]	2.3 ± 0.3[x]	1.7 ± 0.3[x]	0.7 ± 0.1[y]
Activity/intestine[c]	36.1 ± 5.1[x]	25.6 ± 4.0[y]	9.8 ± 0.6[z]

[a] Data represents means ± SEM. Data with significant differences ($P < 0.05$) show a different superscript.
[b] Nanomoles of 3-hydroxybenzo(a)pyrene produced per 30-min assay/mg microsomal protein.
[c] Specific activity × total microsomal protein per intestinal segment.

studies show that a deficiency of sulfur amino acids alone can reduce MFO activity. It is impossible to extrapolate the results obtained from methionine and cysteine deficiencies to other indispensible amino acids. Prior studies have shown that specific amino acid deficiencies cause alterations in hepatic MFO activity unique to the amino acid. Although a 7-day tryptophan deficiency decreased hepatic cytochrome *P*-450 by 40% a similar deficiency of isoleucine or valine produced no significant changes (23).

Summary. Intestinal and hepatic mixed function oxidase (MFO) activity was studied in rats fed sulfur amino acid free diets. Male, Sprague–Dawley rats (~140 g) were randomly assigned to three diets containing L-amino acids as the only source of nitrogen and fed for 4 days. The treatments were the control diet fed *ad libitum* (C), the control diet minus methionine and cysteine (M), and the control diet pair fed (P) with the M-deficient partners. There were no significant differences in total microsomal protein in the first 25 cm of the small intestine but total liver microsomal protein was depressed by decreased food intake and by the sulfur amino acid deficiency ($P < 0.05$). Intestinal aryl hydrocarbon hydroxylase (AHH) specific activity (nmol 3-OH BP/30 min/mg) was (C) 2.30 ± 0.27, (P) 1.75 ± 0.28, (M) 0.65 ± 0.06, and hepatic AHH specific activity

was (C) 15.9 ± 2.1, (P) 16.3 ± 2.4, (M) 4.6 ± 1.3. The data show that sulfur amino acid deficiency causes a similar reduction in intestinal and hepatic MFO activity.

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ergoline and Other Peipheral Serotonin Antagonists Inhibit Prolactin Secretion through Mechanisms Unrelated to Serotonin (40621)[1]

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hysiological stimulatory role for sero- on human prolactin secretion has been ted on the basis of studies in which the olic precursors of serotonin, trypto- and 5-hydroxytryptophan (5-HTP) hown to increase serum prolactin con- ions significantly (1–3). In clinical involving normal individuals and pa- with hyperprolactinemia and acromeg- rotonin receptor antagonists such as oline and methysergide were shown to se the secretion of prolactin (4–9). observations have been interpreted as ents favoring a positive serotonergic n normal and pathological prolactin on in man (4, 8). In this study we t evidence that certain serotonin recep- agonists inhibit prolactin secretion via t effect on the dopamine receptor of pituitary lactotroph. Since the regu- of prolactin secretion in the rat is sim- that in man, these observations support cept that normal and pathological pro- secretion in man is principally regu- by dopamine and dopamine agonists), the role of serotonin being unproven.

hods. Male or female Sprague–Dawley eighing 200–220 g obtained from Flow atories in Dublin, Va. were used for studies. Four flasks each containing emipituitary glands were incubated in issue culture medium 199, containing [4,5-^{3}H]leucine (30 Ci/mmole) for 5 n atmosphere at 95% O_2, 5% CO_2 in a off shaker at 37°C. Aliquots of pituitary enate and incubation medium were ted to polyacrylamide gel electropho- s described in (14). The location of the synthesized [^{3}H]prolactin on the gels was ascertained by subjecting NIAMDD reference prolactin RP-1 to the same electrophoretic separation. The segments of the gels containing newly synthesized prolactin were placed in scintillation vials containing 1 ml of NCS (tissue solubilizing agent; Amersham Searle)–water solution (92.8; vol/vol). A toluene-based scintillation fluid was added to each vial at room temperature and the samples were counted in a liquid scintillation spectrometer.

Other studies utilized females rats (Wistar–Furth, 200–220 g, obtained from ARS/Sprague–Dawley, Madison, Wis.). The MtTW15 tumor which secretes prolactin and growth hormone was transplanted in groups of these rats as described previously (13). Six to eight weeks elapsed before the tumor measured approximately 8 cm^2, at which time the animals were sacrificed.

Prolactin in the serum and in the incubation medium was measured by radioimmunoassay using materials and protocols supplied by the NIAMDD Rat Pituitary Hormone distribution program. All results are expressed as mean ± SEM and statistical analysis was performed by analysis of variance.

Results. The injection of male rats with reserpine increased serum prolactin values 300% (Table I). The parenteral administration of 1 mg/kg metergoline to these animals, however, inhibited significantly ($P < 0.01$) the reserpine-mediated stimulation of prolactin secretion.

The *in vitro* effect of metergoline on prolactin synthesis and release was investigated using normal female rats. The data presented in Fig. 1 demonstrate that 1 μM metergoline inhibited the secretion of newly synthesized [^{3}H]prolactin by 44% ($P < 0.01$) and RIA prolactin by 25% ($P < 0.05$). This inhibitory effect of metergoline was completely overcome by the addition of the dopamine receptor antagonist, haloperidol (10 nM). At the

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indicated concentration haloperidol alone was without influence on prolactin secretion.

The injection of methysergide (25 mg/kg i.p. for 3 days) to six rats bearing the prolactin-secreting pituitary tumor MtTW15 caused a decrease in serum prolactin from 10.7 ± 4.2 to 3.97 ± 0.59 μg/ml. The injection of haloperidol (1 mg/kg daily for 3 days) to six tumor-bearing rats injected with methysergide moderated the suppressive effects of methysergide on serum prolactin significantly, 6.25 ± 0.76 μg/ml ($P < 0.01$).

The effect of several other ergot derivatives with known serotonin antagonist activity on prolactin secretion by the pituitary gland *in vitro* is shown in Table II. A significant direct inhibitory effect of methergine, the metabolic product of methysergide, and of LSD on the secretion of both radioimmunoassayable and newly synthesized [^{3}H]prolactin was observed, while 2-Br-LSD had a weaker inhibitory action on prolactin secretion. The *in vitro* inhibitory effect of LSD on the *in vitro* secretion of prolactin was examined and the results are presented in Fig. 2. These results showed that 1 μ*M* LSD caused a 53% inhibition ($P < 0.01$) in RIA prolactin and an 80% decrease ($P < 0.01$) in [^{3}H]prolactin secretion. Complete blockade of the *in vitro* LSD-mediated inhibition of prolactin secretion was observed by coincubation with 100 μ*M* haloperidol (Fig. 2).

Discussion. Ergot alkaloids, such as ergotamine, ergocryptine, and ergonovine, are nat-

TABLE I. THE INHIBITORY EFFECT OF METERGOLINE ON THE RESERPINE-MEDIATED INCREASE IN SERUM PROLACTIN[a]

	Serum prolactin (ng/ml)
Control	8.9 ± 2.5
Reserpine treated	27.5 ± 6.0*
Reserpine + metergoline treated	7.4 ± 1.7*

[a] Male rats were injected i.p. with reserpine 2 mg each at 10:00 PM and metergoline (1 mg/kg) the next morning at 9:00 AM. Blood was collected at 10:00 AM. All values are mean ± SEM ($n = 6$).

* $P < 0.01$ versus controls.

** $P < 0.01$ versus reserpine group.

TABLE II. THE EFFECT OF ERGOT DERIVATIVES ON PROLACTIN SECRETION BY NORMAL FEMALE PITUITARY GLANDS *in Vitro*[a]

	Incorporation of [^{3}H]leucine into prolactin (cpm/mg pituitary gland)	Prolactin measured by radioimmunoassay (μg/mg pituitary gland)
Control	10,825 ± 540	13.81 ± 0.54
Methergine 1 μ*M*	600 ± 150*	3.97 ± 0.54*
LSD 500 n*M*	4,650 ± 750*	9.14 ± 0.82*
2-Br-LSD 1 n*M*	7,630 ± 190*	13.03 ± 0.83

[a] Four flasks per group each containing three hemipituitary glands; mean ± SEM.

* $P < 0.01$ versus controls.

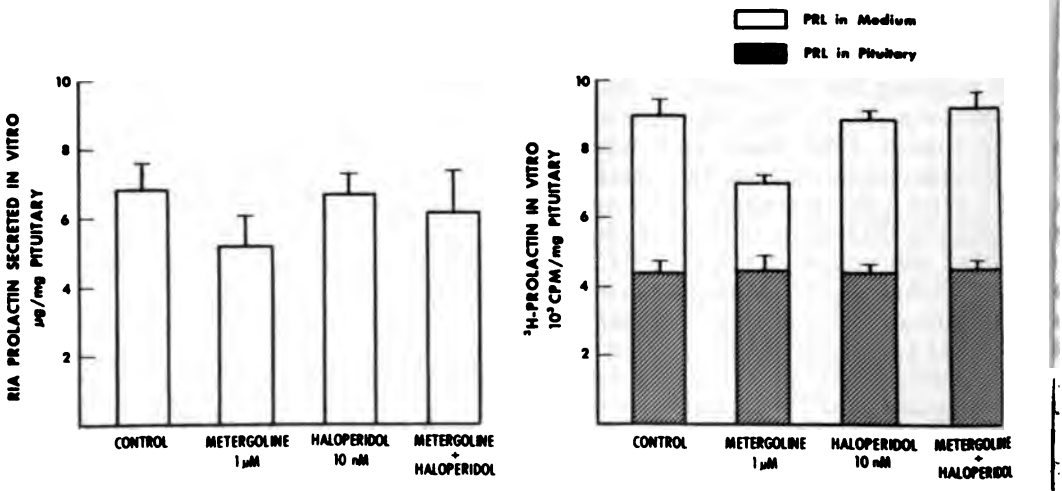
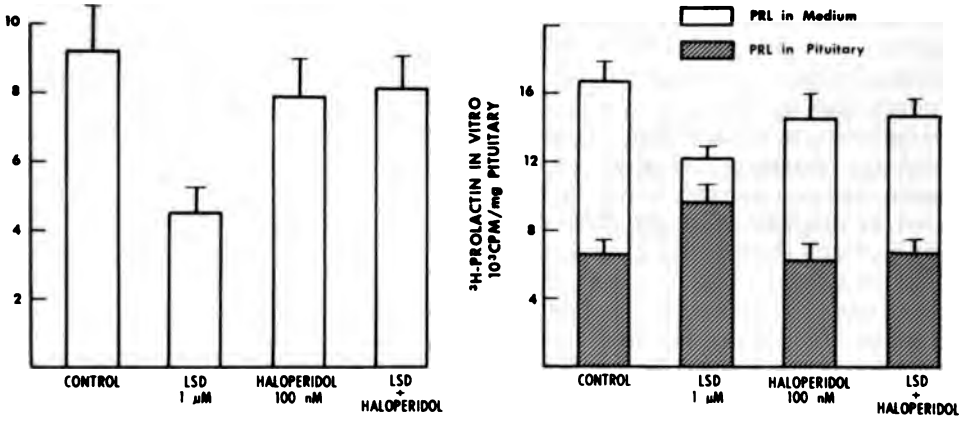


FIG. 1. Effect of haloperidol on metergoline-mediated inhibition of prolactin secretion *in vitro*. Each group contained four flasks containing three hemipituitaries incubated in medium 199 containing the indicated amounts of metergoline and haloperidol. The amount of newly synthesized or radioimmunoassayable prolactin in the pituitary and incubation medium was measured



2. Effect of haloperidol on LSD-mediated inhibition of prolactin secretion *in vitro*. Each group contained ıks containing three hemipituitaries incubated in medium 199 containing the indicated amounts of LSD and dol. The amount of newly synthesized or radioimmunoassayable prolactin in the pituitary and incubation was measured.

occurring substances originating in the Claviceps (Purpurea) (15). Semisynhalogenated alkaloids have been de- d (e.g., 2-bromo-α-ergocryptine) which ss normal and pathological prolactin on via the drugs' dopamine agonist y localized at the pituitary level (16, veral ergot alkaloids and related com- s were early recognized as having pe- al tissue antiserotonin activity, partic- on smooth muscle. In this group, lys- ıcid derivatives such as diethylamide , 2-bromo-LSD, 1-methyl-D-lsyergic utanolamide (methysergide), and 1-D- c-acid butandamide (Methergine) are ally potent (18). The parenteral admin- on of LSD and methergine were re- earlier to suppress prolactin secretion rat (19, 20). In addition, a new class of ounds, the ergoline derivatives, lisuride, ile, and metergoline, has been devel- Metergoline was originally proposed as ıly potent, long-lasting, and selective onist of both central and peripheral ser- receptors (21, 22). It has inhibitory on prolactin secretion in normal indi- s (4), it blocks prolactin secretion in- by breast stimulation during the first rtum week (5), and when administered 24 hr after delivery and continued for s, metergoline was reported to have ory effects on puerperal lactation in a r similar to that elicited by bromocrip- 3). Metergoline inhibited both growth hormone and prolactin release in acromegalic patients (6), but this effect could be prevented by pimozide (7). Methysergide has been reported to suppress the sleep related prolactin rise in normal individuals (24) and to inhibit prolactin levels in normal individuals in and amenorrheic women with hyperprolactinemia (8, 9). This prolactin-lowering effect of methysergide was abolished by sulpiride administration (9). These observations were interpreted by some (4–6, 8) as evidence for a direct antiserotonin-related lowering of prolactin secretion, although others (7, 9) suggested the possibility of a dopaminergic activity of both metergoline and methysergide on prolactin secretion.

A direct, dopamine-agonistic action of all tested ergot derivatives with so-called antiserotonin activity (LSD, 2 Br-LSD, methergine, metergoline) was shown in this study on prolactin secretion by the pituitary gland *in vitro*. In this respect the action of these drugs does not differ from that of dopamine (10). Serotonin itself has been shown to be without direct effect on prolactin secretion by the pituitary gland (25). The mechanism of action of methysergide on prolactin secretion turned out to be more complex. We showed that the compound itself has antidopaminergic activity on prolactin secretion by the pituitary gland *in vitro*; while its prolactin-suppressing effect is probably mediated by a dopamine-agonistic action of its main metabolite, methergine (25). These observations are supported

by the blockade caused by administration of haloperidol on the methysergide-mediated suppression of plasma prolactin in the pituitary tumor-bearing rats.

It is becoming more clear that the generally established stimulatory role of serotonin on prolactin secretion in man is not as firmly founded as originally thought. The earlier reports in which intravenous or oral administration of L-tryptophan and 5-HTP were shown to stimulate prolactin secretion (1–3) have not been confirmed despite a thorough investigation of dosage and time of administration (26–29). As shown in this and our earlier studies (25), the so-called specific serotonin antagonists metergoline, methergine, LSD, and methysergide exert their prolactin secretion suppressive effect via a direct agonistic action on dopamine receptors in the pituitary gland. The results of our studies do not support the suggested role of serotonin in prolactin secretion, but rather support the hypothesis that prolactin secretion is principally regulated by dopamine (10–12).

Summary. The mechanism through which metergoline and other peripheral serotonin receptor antagonists inhibit prolactin secretion was studied. Reserpine, an agent well known to deplete brain catecholamines and serotonin, increased the serum prolactin concentration in male rats. The concomitant injection metergoline completely prevented this increase in hormone level, suggesting that metergoline has a direct effect to inhibit secretion of the hormone. Direct addition of metergoline to pituitary glands incubated *in vitro* decrease the amount of prolactin secreted, confirming this suspicion. Haloperidol at the concentration used was without effect on prolactin secretion, but it completely blocked the metergoline-mediated inhibition of prolactin secretion. Similar effects were observed with methergine, a metabolite of methysergide, and with LSD. These data directly related to the finding that methysergide significantly decreased the *in vivo* prolactin secretion by a transplanted pituitary tumor. Simultaneous injection of haloperidol blocked the inhibitory action of methysergide. Similarly, the LSD-mediated inhibition of prolactin secretion *in vitro* was blocked by haloperidol. We conclude that the inhibiting action of metergoline and methysergide to inhibit prolactin secretion is exerted directly at the pituitary through the stimulation of a dopamine receptor.

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A Small Fraction of Cells Communicates the Maximal Interferon Sensitivity to Population (40622)

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It is generally accepted that the action of interferon results from a direct effect of the molecule on each responding cell. This concept has recently been questioned by our demonstration of the transfer of interferon-induced viral resistance between both heterologous cells (1, 2) and human cell lines (3). This phenomenon required close cell proximity and is thought to represent cellular communication which may be mediated by a secondary messenger molecule(s) (4). Based on these findings, we have proposed that by means of this transfer process the natural mechanism of interferon protection may include action on cells not in contact with interferon but near the interferon-responding cell. Consistent with this idea was our predicted finding that the rate and degree of interferon action was determined by the cell density (5); as the cell density was lowered interferon action decreased and reached a minimum when the majority of cells were not in contact with one another. One explanation is that the most sensitive cells in a heterogeneously responding population determine the overall response only under conditions of high cell density where they contact less sensitive cells. This idea has been tested by cloning cells to determine the response of the individually reacting cells and their contribution to the response of the whole culture. We found a marked heterogeneity between cells in their sensitivity to interferon and their ability to transfer resistance. Reconstitution experiments indicate that only a minor fraction of cells determines the interferon response of the population.

Materials and methods. Cell culturing and cloning. Mouse L-929 cells in Eagle's minimal essential medium (MEM) with 10% fetal calf serum were passaged 1 to 10 biweekly. For cloning, L cells were suspended in MEM supplemented with 10% fetal calf serum, nonessential amino acids, and 25 m*M* Hepes buffer. Cells (20/ml) were distributed at 5 ml/flask into a series of Falcon tissue c flasks (area, 25 cm^2). They were inc undisturbed at 37° for 10 days at whic the medium was replaced with mouse feron or fresh medium. Following inte treatment, 10^5 plaque-forming units (P vesicular stomatitis virus (VSV) was ad each flask (approximate input multipli infection was 20 PFU/cell). After ov incubation at 37°, surviving clones stained with crystal violet and counte cloning efficiency of L cells was 70–10

For the establishment of cloned lines, 0.1 ml/well of a cell suspensi cells/ml) was added to each well of Micro Test II tissue culture plates. A days incubation in 4% CO_2 at 37°, containing a single clone were visuall tified. About 4 days later, the cells fron wells were removed with trypsin and in Falcon tissue culture flasks (25 cm^2 weeks then into 75-cm^2 flasks. Thre later they were assayed for interferon tivity. Highly resistant or sensitive cells were then used in cell mixing e ments.

Interferon. Interferon was produced fection of mouse L cells with a lent strain of Newcastle disease virus (NI previously described (6).

Sensitivity of L-cell clones to inte was determined either by a microyield tion assay (1×10^5 cells/well) (1) or a modification of the method of micro reduction using methylcellulose overl stead of carboxymethylcellulose (7.5 cells/well) (7). The VSV challenge d these two assays was 3 PFU/cell and PFU/well, respectively. Interferon tit expressed in terms of the NIH resear erence mouse interferon as assayed c parental L-929 cells.

Results. Heterogeneity of the respo interferon. Cell cloning was employed tablish whether there is variation in the

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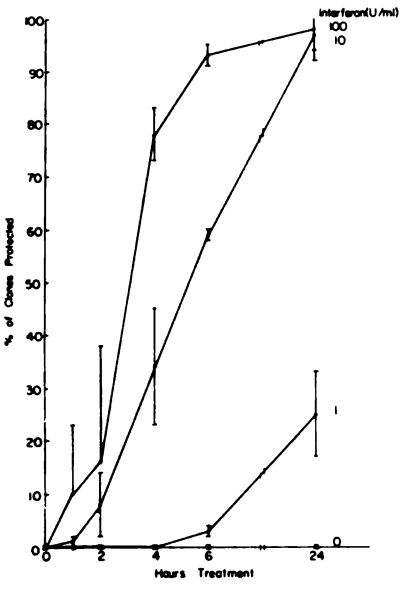
ı response of individual L cells within a ılation. Figure 1 shows that when a low ity of L cells is plated such that each cell ıs a clone, the number of clones protected nst virus challenge increased with the th of interferon treatment. Likewise, at a n time, there is an increase in the number rotected clones with increasing concentra- s of interferon. These data show that vidual L cells differ in the amount of rferon to which they respond as well as time required for the response. These ings strongly suggest marked differences he interferon response of individual L

o determine the degree of variation, cell s were established from individual L cells. resentative dose–responses for three of e cloned L-cell lines are shown in Fig. 2. he extremes, clones differed by 10-fold in r sensitivity to interferon and almost 100- in the maximum degree of protection nst virus. Table I summarizes the results ıe interferon responses of 18 L-cell clones. ırly, there is a large heterogeneity among vidual L cells in both the sensitivity to maximum protection afforded by inter- n.

ransfer of interferon-induced viral resist- between cloned L cells. If the transfer of



IG. 1. Variation in interferon protection of L-cell s against VSV.

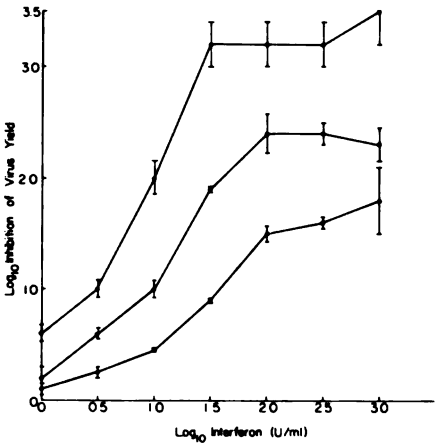


FIG. 2. Representative interferon dose–responses of three cloned L-cell lines. Data are presented as the mean (three replicates) and 95% confidence limits.

TABLE I. VARIATION OF THE INTERFERON DOSE–RESPONSES OF CLONED L CELLS

Clone	Interferon dose (U/ml) $\log_{10}$ inhibition of VSV yield[a]					
	1	3	10	30	100	300
A	0.03	0.19	0.37	0.92	1.5	1.6
B	0.20	0.26	0.76	1.5	1.7	1.9
C	0.08	0.88	1.5	2.6	2.7	2.9
D	0.03	0.74	1.8	2.3	2.5	2.8
E	0	0.70	1.8	2.5	2.5	2.8
F	0.09	1.2	1.7	2.6	2.8	3.3
G	0.20	0.45	1.5	1.8	2.1	1.9
H	0.07	0.68	1.7	2.8	2.8	2.6
I	0.10	0.28	0.92	1.5	2.3	2.2
J	0.10	0.85	2.0	3.2	3.2	3.3
K	0	0.44	1.5	2.2	2.6	2.4
L	0	0.43	1.2	1.9	2.2	2.2
M	0.42	0.88	2.0	2.7	2.8	73.5
N	0	0.17	1.2	1.8	2.2	2.5
O	0	0.4	1.0	1.9	2.4	2.4
P	0.36	0.78	1.8	2.3	2.3	2.5
Q	0.15	1.2	1.9	2.4	2.6	2.5
R	0	0.47	1.1	1.9	2.5	2.5

[a] Differences of 0.5 $\log_{10}$ are significant at $P < 0.05$.

viral resistance occurs between individual cells of a cell line, then this should be demonstrable with cloned cells derived from the parent population.

When cloned L cells of "high" (clone 2) and "low" (clone 1) sensitivity to interferon were cocultivated in a 1-to-1 ratio the response of the mixed population approached that of the "high" responding clone (Fig. 3). The virus yields from noninterferon-treated cells of "high" and "low" responding clones

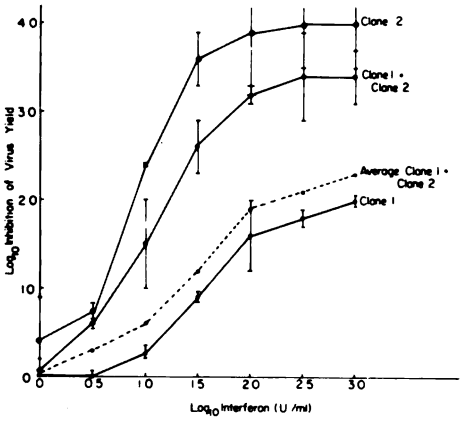


FIG. 3. Transfer of interferon-induced viral resistance, between cloned L cells. Data are presented as the mean (three replicates) and 95% confidence limits.

(alone or mixed) were the same and the yield from interferon-treated "high" responding cells was insignificant compared to equivalently treated "low" responding cells. Thus if transfer had not occurred, in the presence of interferon the yield should have been 50% of the yield from interferon-treated "low" responding cells. In terms of the maximum amount of protection confered by interferon, there was no significant difference between the mixed cells and the "high" responding cells. This observation has been repeated with three different clones. The increase in sensitivity ranged from 3- to 30-fold and the increased maximum degree of protection ranged from 5- to 30-fold. Based on these data, it seems that individual cells within a heterogeneous population can transfer interferon-induced viral resistance amongst themselves.

The fraction of cells which determines the interferon response of the population. Since the preceding experiments demonstrated that transfer of viral resistance can occur within a population of cells, it is important to determine what fraction of cells controls the response of the population. This was investigated by comparison of the interferon response of the parent L-cell population, 40 L-cell clones, and a reconstituted L-cell population. In a plaque reduction assay, the response of the reconstituted population (equal numbers of each cloned cells) was not significantly different from the parental population, while both were four to five times more sensitive than the average of the 40 individual clones (Fig. 4). This indicates that transfer occurs within L cells and can be demonstrated with a plaque reduction assay. It also appears that the reconstituted population closely approximates the parental population.

A compilation and analysis of this data (Table II) shows that with 1 and 3.3 U/ml of interferon the response of the population is determined at most by 10 and 30% of the cells, respectively. Whether all of these clones transfer is not known at present. At 10 and 33 U/ml almost all the cells respond equally (100% inhibition). Thus, the transfer of viral resistance can play an important role in the action of interferon.

Discussion. We have proposed that the natural action of interferon does not require a direct effect of the molecule on each cell. This proposition stemmed from our previous demonstrations of the transfer of interferon-induced viral resistance between cells (1–3). Supportive of this was the finding that interferon action was determined by the cell density (5). At a cell density where the majority of cells in a population could not contact one another there was a precipitous drop in inter-

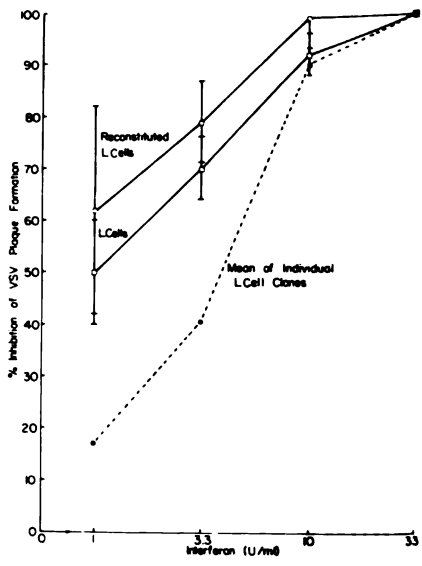


FIG. 4. Interferon dose-responses of "parental" L cells, a reconstituted L-cell population, and the mean response of 40 L-cell clones. Data for the "parental" L cells and the reconstituted L-cell population are presented as the mean (three replicates) and 95% confidence limits.

LE II. THE FRACTION OF CELLS WHICH DETERMINES THE INTERFERON RESPONSE OF THE POPULATION

feron ml)	Percentage inhibition of VSV plaque number, number of clones			Percentage inhibition of VSV plaque number		Percentage of clones at or above reconstituted population's percentage inhibition
	0–35	31–60	61–99	Mean of clones	Reconstituted population	
	35	1	4	17	62	10
1.3	25	3	12	40	79	30
)	2	2	36	90	99	78
)	1	0	39	98	100	98

ctivity. This cell-proximity effect was ed to result from variation in inter-ensitivity between individual cells in ulation and an inability of the most e, first-responding cells to transfer ral resistance to less sensitive, slower-ling cells when they were not in con-

is study is was shown by the cloning vidual L cells that there is a very l heterogeneity among individual cells ı their sensitivity to and maximum of protection afforded by interferon. r, cloned L cells of "high" interferon ity can transfer their viral resistance es of "low" sensitivity. By studying the on response of individually reacting and a reconstituted parental popula-cells, it was found that as few as 10% e cells can determine the response of ulation. These findings strongly sup-ır interpretation of the cell proximity ınd our contention that the action of on does not require a direct effect of lecule on each responding cell.

earlier demonstrations of the transfer rferon-induced viral resistance em-cells of different animal species (1, 2). bservation of transfer among cells a population from a single species s that the transfer process is opera-*n vivo*. This is all the more likely since proximity effect occurs with primary embryo cells as well as diploid human ısts (which are similar to normal cells (5). This means that the natural mech-of interferon protection probably in-action on cells near the interferon-ling cell. This process would amplify rferon response since sensitive, fast-ling cells would transfer resistance to sitive, slower-responding cells which ıte the bulk of the population. The observation that a small fraction of cells (10%) determines the response of the population shows that the transfer process plays a highly important role in the action of interferon.

There are many similarities between interferon and polypeptide hormones (8). For instance, interferon acts at the cell membrane (9, 10). It has been proposed that, as with polypeptide hormones, the interferon-cell membrane interaction produces a secondary messenger molecule(s) which induces the antiviral state (1, 11–13). Based on our previous data a likely mechanism for the transfer of interferon-induced viral resistance may be gap junctional transfer of the putative secondary messenger(s). Shortly after our demonstration of cell-to-cell communication of interferon activity, a similar finding with polypeptide hormones was reported (14). This leads to the intriguing possibility that as with interferon there may be marked heterogeneity among "sensitive" cells in their response to polypeptide hormones and only a small number of cells determine the response of the population (or *in vivo* the tissue). If this is correct then direct cell-to-cell communication represents a novel mechanism for the amplification of hormone or hormone-like activity.

Summary. Cloned L cells were shown to be very heterogeneous in their time and dose-responses to mouse interferon. Sensitive cells transferred interferon-induced viral resistance to less sensitive cells making them equally sensitive. A small fraction of cells was found to determine the overall response of the "parental" L-cell population. Based on these findings a new mechanism of interferon action on cells not in contact with interferon was proposed. The implications of this proposal to polypeptide hormone action are discussed.

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ıct of Bovine Testicular Extracts on Plasma Gonadotrophins of X-Irradiated Rats (40623)[1]

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ırdloff *et al.* (1) provided indirect evi- that the germinal epithelium of the was important in the negative feedback ıl of FSH secretion in the rat. More ly, other investigators (2–5) have di- demonstrated the presence of a water- e testicular factor which prevents the stration rise of FSH in the male. This was named inhibin by McCullagh (6). ive destruction of the germinal epithe- ıy X rays (7), experimental cryptorchid-), drugs (8), or ligation of the ductus ıtes (9) induced an increase of blood of FSH, but not of LH. We have em- l the X-irradiated rat model, referred to toli Cell Enriched or SCE (10), to dem- te that an inhibitory factor (inhibin) ned in crude bovine testicular extracts can suppress the elevation of FSH and ıonstrate the probable cell of origin of n.

erials and Methods. Bovine testicular ts (bTE) were prepared as previously bed (2), and bovine kidney extracts were similar prepared and used as ecific tissue controls. The protein con- tions in the preparations were deter- by the Folin–Lowry porcedure (11) ere between 950 and 980 mg/g of ly- zed testicular extract.

determine the effects of tissue extracts ısma gonadotrophins, the 34-day-old lectomized rat model (2) was used. Immature male rats (34 days) were castrated at 2000 hr. At 0800 hr of Day 35 a blood sample was drawn by a cardiac puncture, and the animal was injected s.c. with vehicle or tissue extract in vehicle. At 2000 hr on Day 35 (24 hr after castration and 12 hr after injection) the animals were exsanguinated, and the plasma was stored at −20° for subsequent gonadotrophin determination.

Male, Sprague–Dawley rats were maintained on a controlled light schedule (12 L:12 D) with free access to food and water in a temperature-controlled room. Thirty-four-day-old rats, whose upper bodies and heads were shielded by lead, received a dose of 500 rads of X rays from a General Electric Maxitron 300 (Tungsten Filament). Nonirradiated littermates were used as controls. Forty-nine days later (postnatal Day 83), blood samples were collected by cardiac puncture under light ether anesthesia. All rats were injected s.c. once daily for 3 days with either 4% gelatin or 250 mg of bTE in 4% gel. Twenty-four hours after the last injection, all rats were castrated and treatments were continued for 3 more days, and on the 4th day after castration, all rats were exsanguinated and the plasmas were frozen for subsequent hormone analysis. The testes, ventral prostates, and seminal vesicles were excised and weighed, and the right testis of each rat was fixed for histological examination.

Plasma concentrations of FSH and LH were measured with the rat radioimmunoassay (RIA) kits generously provided by the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD). The intraassay variability for each assay was less than 10%, and, since all bloods were run in the same assay, there was no interassay variability. The assays were run at 37° for 36 hr as described by Moudgal and Madhwa Raj (12). The highly purified NIAMDD-LH and NIAMDD-FSH were labeled with ^{131}I by the

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s study was presented in part at the 9th Annual ; of the Society for the Study of Reproduction in lphia in 1976.

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Chloramine-T method of Greenwood *et al.* (13).

All data were statistically evaluated by a one-way analysis of variance (ANOVA).

Results. In a preliminary study, the biological activity of bTE was demonstrated in 34-day-old, orchidectomized rats (2) with doses ranging from 50 to 250 mg. The postcastration rise of FSH was completely suppressed and that of LH was only partially suppressed (Table I). There was a significant increase of plasma FSH ($P < 0.01$) 12 h after castration (gp 2) as compared to the intact controls. Twelve hours after injection of gel (24 hr after castration) into the castrate controls (gp 2), there was a 47% increase ($P < 0.01$) in plasma FSH levels and a 140% increase ($P < 0.001$) in plasma LH levels. Group 3 rats were administered 100 mg of bTE per rat. These animals exhibited essentially unchanged (−3%) plasma FSH levels (12 vs 24 hr) and a 34% increase ($P < 0.001$) in plasma LH levels. Thus, bTE totally prevented further postcastration rise of plasma FSH and partially prevented the rise of LH. FSH and LH levels in group 4 rats which received 100 mg of bKE did not differ from the castrate control group.

The selective destruction of the germinal epithelium reduced rat testicular weights to approximately 33% of intact control rat testicular weights (Table II). There were no significant differences in the ventral prostate weights among the groups (Table II), although the slight decrease in weights of the SCE groups may be due to some radiation damage to the interstitial cells. There were also no differences in seminal vesicle weights, which were omitted from the data since they did not contribute additional, relevant data.

In addition to the X-irradiation-induced destruction of the germinal epithelium there was a more than two-fold increase of plasma FSH over the intact controls (gps 1 and 2, Table II). In the X-irradiated rats given bTE (gp 3), plasma FSH was suppressed ($P < 0.025$) to the level of intact controls. Although there were no changes in plasma LH levels over the initial treatment period, there was an increase ($P < 0.05$) in LH due to X irradiation. Both gonadotrophins rose in all rats as a result of castration on Day 4. However, continued bTE treatment for 3 days resulted in a 67% suppression of plasma FSH and a 33% suppression of LH as compared to castrate controls (gp 2 vs gp 3, Table II). In a

TABLE I. EFFECTS OF BOVINE TESTICULAR EXTRACTS AND BOVINE KIDNEY EXTRACTS ON PLASMA FSH AND LH IN 34-DAY-OLD CASTRATE MALE RATS[a]

Group	Treatment (No. of animals)	FSH (ng/ml)[b] 12 hr	FSH (ng/ml)[b] 24 hr	LH (ng/ml)[b] 12 hr	LH (ng/ml)[b] 24 hr
1	Intact (4)	383 ± 20*	439 ± 21*	49 ± 7*	66 ± 21*
2	Castrate (6)	525 ± 68*	771 ± 71*	148 ± 57*, *	358 ± 37**
3	Castrate + bTE (6)	582 ± 17*	573 ± 18*	113 ± 3*, **	167 ± 5*, **
4	Castrate + bKE[c] (7)	531 ± 22*	675 ± 37*	161 ± 23*, **	428 ± 72**

[a] Injected (s.c.) 8% gelatin, 100 mg bTE in 8% gel, or 100 mg bKE in 8% gel at 12 hr postcastration. All rats exsanguinated 12 hr after the injections (= 24 hr postcastration).

[b] $\bar{X} \pm$ SEM.

[c] bKE was prepared similarly to bTE.

* $P < 0.01$.

** $P < 0.001$.

TABLE II. EFFECT OF BOVINE TESTICULAR EXTRACTS ON X-IRRADIATED RATS[a]

Group	Treatment (no. of rats)[b]	Testes weight (mg)[c]	Ventral prostate weight	FSH (ng/ml)[c] 0 hr	FSH (ng/ml)[c] Day 4	FSH (ng/ml)[c] Day 8	LH (ng/ml)[c] 0 hr	LH (ng/ml)[c] Day 4	LH (ng/ml)[c] Day 8
1	Intact control (6)	3695 ± 67[m]	429 ± 56	383 ± 30[f]	270 ± 18[f]	1942 ± 261[h]	51 ± 8[g]	49 ± 4[g]	453 ± 49[m]
2	SCE (7)	1188 ± 64[h]	427 ± 27	873 ± 54[m]	879 ± 32[m]	2240 ± 324[h]	93 ± 7[i]	100 ± 11[i]	504 ± 75[m]
3	SCE + bTE (9)	1352 ± 64[h]	424 ± 36	819 ± 49[m]	351 ± 30[f]	1283 ± 135[i]	92 ± 14[i]	94 ± 6[i]	285 ± 35[n]
4	SCE + bKE (7)	1324 ± 108[h]	408 ± 27	884 ± 75[m]	968 ± 90[m]	2392 ± 314[h]	88 ± 18[i]	84 ± 16[i]	472 ± 34[m]

[a] Rats were irradiated on postnatal Day 34 and all injections were begun on postnatal Day 83.

[b] Received 1 ml 4% gelatin or 250 mg bTE/ml 4% gel, or 250 mg bKE/ml 4% gel, s.c., once a day for 3 days, castrated on Day 4, and after another 3 days of once daily injections were exsanguinated on Day 8.

[c] $\bar{X} \pm$ SE. $P < 0.05$ = *k, l.* $P < 0.025$ = *e, g; h,i; m, n.* $P < 0.005$ = *e, g; g, h; e, i; l, n.* $P < 0.001$ = *a, b; e, h; k, m.*

ary study (unpublished), 3 days of
ıtment (40 mg) in intact rats selec-
ɔcreased plasma FSH levels ($P <$
ıd had no effect on plasma LH, tes-
/eights, or ventral prostate weights.
micrographs of testicular sections
rated the effects of X irradiation on
ıinal epithelium. In the X-irradiated
› spermatozoa, spermatids, or sper-
ɔs were seen. Only stem cells sper-
ıia, and Sertoli cells were present.
peared normal according to cell size,
ıd nuclear structure.

sion. There have been a number of
rations that selective suppression of
ı be achieved with testicular extracts
minal plasma (3), and rete testicular
in castrate males. In this study, using
ıl model with selective destruction of
ninal epithelium by X irradiation,
esticular extracts suppressed plasma
ıonirradiated levels and had no effect
X-irradiation-induced elevation of
.H ($P < 0.05$). The rise of plasma LH
ggests that some damage to the inter-
lls had occurred. In response to cas-
he bTE prevented the rise of plasma
l LH to untreated castrate rat plasma
he bTE consistently suppressed FSH,
r at very large doses suppressed LH
ıted or X-irradiated, immature rats
ryptorchid rats (preliminary study).
:idney extracts in this model had no
FSH or LH.

bility of the bTE to selectively sup-
sma FSH may seem to be equivocal
udy. However, both a specific effect
only and effects on FSH and LH
ın noted with different testicular ex-
d experimental protocols in our lab-
unpublished observations (14)). We
nd (14) that high doses or bTE can
LH, although in the present study
I) this effect was seen only after
t with bTE for 8 days and castration.
ıpression of LH was also observed
gh doses of rete testis fluid were
ered to male rats (15). Effects on
of both gonadotrophins has been
ly noted in the literature ((5), see
n by Swerdloff and Hudson, p. 472
is dual suppression may be due to
of the batch of bTE, dose-adminis-
tered, species of test animal, and/or experimental model employed (for excellent review, see cf. (17)). Recently (18) two models for the regulation of gonadotrophin secretion have been proposed, both of which implicate inhibin and androgens. In the first, androgens control the secretion of FSH and LH, but inhibin controls about one-third of the feedback control of FSH only. In the second model, both inhibin and androgens control both FSH and LH, with inhibin having a greater effect on FSH and androgens more greatly affecting LH. Our data appear to support the second model. However, until inhibin is completely isolated and purified, its effects remain controversial.

It is probable that one of the cellular elements in the seminiferous tubule is the source of inhibin. There is still a question as to whether the Sertoli cell (19, 20), Sertoli-cell/spermatogonial unit (21), or spermatid (22) is the source of inhibin. Destruction of the germinal epithelium by X irradiation leaves only the Sertoli cell and some spermatogonia. If the Sertoli cell or the Sertoli-cell/spermatogonial unit were the source of inhibin, FSH levels should be unaffected by X rays assuming no irradiation damage based on light microscopy. Clinical studies have demonstrated that FSH levels were increased in azoospermic men with testicular histologies of Sertoli cells only or Sertoli cells and germinal cells up to primary spermatocytes, whereas no relationship with FSH levels could be inferred from oligospermic men (5, 22). Baker *et al.* (5) found no significant relationship between FSH levels and Sertoli cell numbers in analyses of cross sections of seminiferous tubules in both fertile and infertile men. These observations, implying germinal cells (spermatid or spermatozoa) as the source of inhibin, are in contrast with those using an *in vitro* Sertoli cell culture system contaminated with less than 20% germ cells (19). Rich and deKretser (20) studied the effect of hydroxyurea (HU) treatment, a chronic vitamin-A-deficient diet, or fetal irradiation on Day 20 of gestation (all of which damaged the germinal epithelium to varying degrees) on serum FSH and Sertoli cell secretory function. Regardless of the amount of germinal cell damage, all treatments were found to impair Sertoli cell secretory func-

tion, as measured by androgen-binding protein (ABP) levels in the testis. Serum FSH levels were increased in all groups. These findings strongly suggest that the Sertoli cell is the source of inhibin. Furthermore, plasma FSH levels were elevated after X irradiation, yet the Sertoli cells appeared to be normal upon observation with the light microscope. However, ultrastructural examination of Sertoli cells, obtained from patients with germinal aplasia, exhibited morphological abnormalities suggestive of impaired (secretory) function (23). These observations coupled with the earlier finding (10) that Sertoli cells may be the *only* target in the testis for FSH strongly suggest that inhibin is produced by the Sertoli cell. Our study does not provide direct evidence for any specific cell type, but lends further support to the hypothesis that the Sertoli cell is the probable cell of origin of inhibin.

Summary. The effect of bovine testicular extracts (bTE) on plasma FSH levels in both castrate and X-irradiated male rats has been examined. Biological activity was demonstrated in 34-day-old, orchidectomized rats with doses ranging from 50 to 250 mg. Testicular weights of X-irradiated rats 49 days after X irradiation were decreased by approximately 67%, whereas the ventral prostate weights were unaffected. Plasma FSH levels (383 ng/ml) increased significantly as a result of X irradiation (873 ng/ml). Plasma LH (51 ng/ml) also rose as a result of X irradiation (93 ng/ml), suggesting that there was some interstitial cell damage or some Sertoli cell damage. Two hundred and fifty milligrams of bTE administered in 4% gelatin given once daily for 3 days suppressed the elevated plasma FSH, but was without effect on plasma LH. Castration and a further 3 days of treatment resulted in a significant postcastration suppression in plasma FSH (control = 1942 ng/ml; bTE = 1283 ng/ml), and resulted in a suppression of LH as well. This LH response (control = 453 ng/ml; bTE = 285 ng/ml) is probably due to the large dose of bTE employed. A bovine kidney extract (bKE) was totally ineffective in suppressing FSH or LH when given at a dose of 250 mg/rat/day in 4% gelatin. These data lend further support to the existence of inhibin, contained in bTE, and to its role as a regulator of FSH in the male. The probable site of origin of inhibin is the Sertoli cell.

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Methanol Potentiation of Carbon Tetrachloride-Induced Hepatotoxicity (40624)

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It has been established that aliphatic alcohols are capable of potentiating CCl_4 hepatotoxicity (1–5). The degree of potentiation has been shown to vary considerably from one aliphatic alcohol to another. Traiger and Plaa (5) showed this variability in mice, using serum glutamic-pyruvate transaminase (SGPT) as an index of toxicity. Pretreatment with methanol, ethanol, isopropanol, *n*-propanol, *n*-butanol, and 2-methyl-2-pentanol showed that only isopropyl, ethyl, and methyl alcohols were significant potentiators in mice. Of the three, isopropanol showed the greatest potentiation, approximately, 7-fold greater than methanol and 20-fold greater than ethanol.

Studies of alcohol potentiation of CCl_4 hepatotoxicity have primarily investigated the effects of ethanol; however, additional studies have been concerned with isopropanol. Little information is available concerning methanol potentiation of CCl_4 hepatotoxicity. To date, methanol potentiation of CCl_4-induced hepatotoxicity has not been investigated *in vivo* in the rat. In addition, the only comparative potentiation study with methanol and other alcohols has been in mice. Similarly, few studies have investigated the mechanism by which alcohols potentiate CCl_4-induced hepatotoxicity. Attempts to uncover the mechanism of ethanol and isopropanol potentiation have been made, but the specific site of action is yet unknown (6). The toxicity of CCl_4 is dependent on its metabolism and probably involves the resultant trichloromethyl free radical which causes lipid peroxidation and/or covalent binding to cellular macromolecules.

Oral pretreatment of rats with ethanol, methanol, and isopropanol has been found to alter hepatic microsomal metabolism of foreign compounds (7). However, all three alcohols do not cause the same alterations. Ethanol, methanol, and isopropanol all increased aniline hydroxylation, but only isopropanol increased aminopyrine demethylation. Ethanol and methanol inhibited aminopyrine demethylation.

The time course of potentiation of CCl_4 hepatotoxicity has been studied with ethanol and isopropanol in rats (5). Maximum potentiation of toxicity occurred when rats were pretreated 18–24 hr prior to CCl_4 administration. A similar time-course study has not yet been performed with methanol.

The purpose of the present investigation was to examine the time course of methanol potentiation of CCl_4-induced hepatotoxicity in rats. Additionally, the relative magnitude of methanol potentiation in rats was compared to that observed with ethanol and isopropanol. In an attempt to determine the mechanism of methanol potentiation, the effect of methanol pretreatment on CCl_4-induced lipid peroxidation and on the irreversible binding of $^{14}CCl_4$ to hepatic microsomal protein and extracted lipids was also investigated.

Materials and methods. General. Male, Sprague–Dawley rats, weighing 200–250 g, were used throughout the study. Before and during the experiment, the animals were maintained on an *ad libitum* diet of commercial chow and water.

Analytical grades of methanol, isopropanol, and ethanol were orally administered at doses approximately equal to ½ of the respective LD_{50} (7.0, 2.5, and 6.0 ml/kg, respectively). These values were derived from similar studies by Cornish and Adefuin (4) and Traiger and Plaa (5). Analytical grade carbon tetrachloride was injected intraperitoneally, dissolved in a corn oil vehicle. Orally administered saline (10 ml/kg) was used as control for alcohol pretreatments. Corn oil (10 ml/kg i.p.) was used as control for CCl_4.

Methanol potentiation time-course study. Methanol was given orally at various times prior to CCl_4 injection (0.1 ml/kg i.p.). Animals were anesthetized with ether 24 hr later

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ood samples were obtained from the aorta with a syringe rinsed in heparin. e aminotransferase (ALT or SGPT) easured in plasma by the method of n and Frankel (8).

parative hepatotoxicity study. Metha- propanol, and ethanol were given 4 hr prior to injection of various doses . Twenty-four hours after CCl_4 injec- its were anesthetized with ether and was taken for ALT activity measure- s described above. Two pieces of liver ien obtained, each weighing approxi- l g. One section was then used to ine hepatic triglyceride levels by the l of Van Handel and Zilversmit (9), as d by Butler *et al.* (10). The other of liver was used to measure hepatic -6-phosphatase activity by the method ed by Klaassen and Plaa (11) except liver homogenate was used and sam- re incubated for 30 min.

osomal fraction isolation. Liver from or methanol-pretreated rats was ho- zed in 4 vol/g liver of Tris–KCl- buffer (20 m*M*–1.15%–3.0 m*M*), pH ie homogenate was centrifuged at for 30 min in a Sorvall Model RC2- ifuge at 4°. The supernatant was re- and centrifuged at 100,000*g* for 65 4° in a Beckman Model L ultracentri- elding a microsomal-rich pellet.

ersible binding of $^{14}CCl_4$ to microsomal and lipid. The microsomal fraction d as previously described was resus- in 4 vol of the same Tris–KCl–EDTA resulting in a protein concentration of /ml. The homogenate was incubated nitrogen at 37° in a total of 8 ml of ion mixture containing a NADPH re- ing system consisting of the following s final concentrations: 2.5 m*M* nico- le, isocitrate dehydrogenase (0.02 l), 0.10 m*M* NADP, 20 m*M* DL-iso- and 5.0 m*M* $MgCl_2$. This system is to that described by Glende *et al.* (12). sealing the reaction flasks with air- ips, the flask and its contents were with nitrogen for 4 min. Radioactive tetrachloride ($^{14}CCl_4$), specific activity i/mmole (New England Nuclear, Bos- ass.), was diluted in absolute ethanol nradioactive CCl_4 such that when 27 μl of the CCl_4–ethanol mixture was injected into the reaction flask, a total of 830 nmole of both radioactive and nonradioactive CCl_4 was introduced. Final specific activity of CCl_4 was 1.5 mCi/mmole.

Irreversible binding of ^{14}C to protein was determined by drawing 1 ml of the incubation mixture into a syringe after 6 min of incubation and immediately injecting the sample into 1 ml of 10% trichloracetic acid. The samples were placed into a boiling water bath for 10 min. After centrifugation at 3000*g* for 15 min and removal of the supernatant, the pellet was resuspended in 5 ml of methanol: ether (3:1), heated to 60° for 10 min, and then centrifuged as before. The resulting supernatant was then removed by aspiration. This washing procedure was repeated six times to assure that any unbound radiolabeled CCl_4 was removed. After the final wash, the remaining protein was dissolved in 1 ml of 1 *N* NaOH and the radioactivity determined by liquid scintillation counting. This procedure is similar to that described by Sipes *et al.* (13).

Irreversible binding of $^{14}CCl_4$ to hepatic microsomal lipids after 6 min incubation was determined by removing 1 ml of incubation mixture and injecting it into 9 ml of chloroform:methanol (2:1). The mixture was shaken for 5 min and then centrifuged at 3000*g* for 10 min. The resulting supernatant was then transferred to a graduated tube and a final volume of 10 ml was obtained by adding the chloroform:methanol (2:1). Two milliliters of distilled water was added and after thorough mixing and centrifugation at 3000 *g* for 10 min, the upper phase was aspirated off and discarded. The remaining lower phase was washed twice with 2 ml of upper phase obtained by mixing 25 ml of distilled water and 95 ml of chloroform:methanol (2:1). The washed lower phase was then transferred to a scintillation vial and evaporated under nitrogen at 40°. This method is similar to that described by Castro and Diaz Gomez (14). Radioactivity was determined by liquid scintillation counting. Protein concentrations of microsomal homogenates were determined by the method of Lowry *et al.* (15). The results are expressed as nanomoles $^{14}CCl_4$ irreversibly bound to microsomal lipid or protein per milligram of microsomal protein.

CCl_4-Induced lipid peroxidation. Rats were

pretreated with either methanol or saline 24 hr prior to CCl_4 injection. Exactly 30 min after CCl_4 administration, the animals were sacrificed and microsomes were obtained as described above. The determination of lipid peroxidation by measuring diene conjugation has been described by Klaassen and Plaa (11). In this study, however, 1 g of liver was homogenized in 3 vol of buffer. Results are expressed as OD units (243 nm), from extracted microsomal lipids obtained from 1 g of liver.

Statistics. Statistical analysis was performed using analysis of variance and Student's *t* test when analysis of variance demonstrated statistical differences (16). The level of statistical significance was chosen as $P < 0.05$.

Results. Figure 1 shows alanine aminotransferase activity (ALT) in plasma of rats pretreated with methanol at various times prior to CCl_4 injection (0.1 ml/kg). Administration of either methanol or 0.1 ml/kg CCl_4 did not produce a significant increase in plasma ALT activity. However, a significant increase in ALT was observed 24 hr following 1.0 ml/kg CCl_4 (insert Fig. 1). The potentiation appeared to be maximal when CCl_4 was given 48 hr after methanol. However, maximum variability also occurred at this time. It should be noted that when rats were pretreated with methanol, 0.1 ml/kg CCl_4 produced a greater elevation of ALT than when a dose of CCl_4 10 times higher was given to control rats.

The effect of pretreatment with methanol, isopropanol, and ethanol on CCl_4 hepatotoxicity assessed by plasma ALT, hepatic accumulation of triglycerides, and hepatic glucose-6-phosphatase activity is shown in Fig. 2. The top panel in the figure depicts the plasma ALT in rats pretreated with the alcohols 24 hr before receiving various doses of CCl_4. In saline-pretreated rats, ALT values were not significantly altered at any dose of CCl_4 administered. Similarly, treatment with

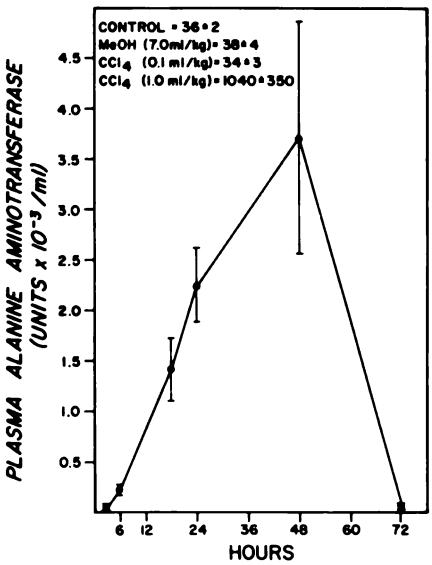

FIG. 1. Plasma ALT in rats pretreated with methanol (7.0 ml/kg p.o.) 24 hr after receiving CCl_4 (0.1 ml/kg i.p.). The abscissa indicates the time interval between methanol pretreatment and CCl_4 administration. The insert indicates the plasma ALT in control rats, and in rats 24 hr after the described treatments.

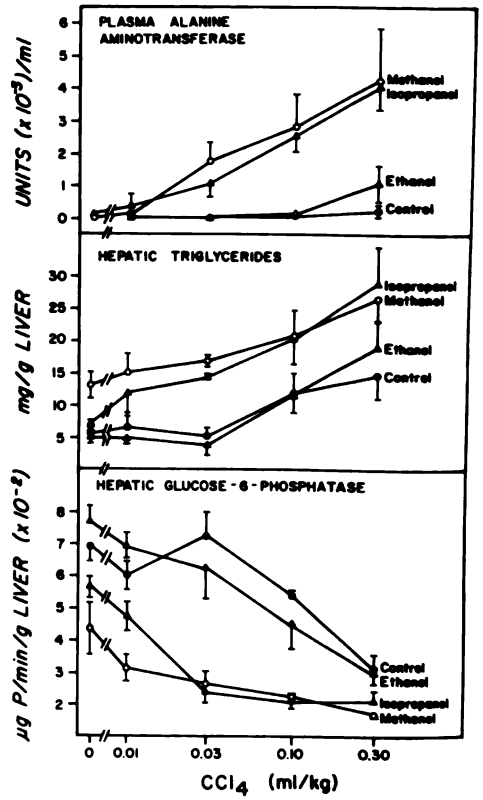

FIG. 2. Assessment of CCl_4 hepatotoxicity using plasma ALT, hepatic triglycerides, and hepatic glucose-6-phosphatase in rats pretreated with methanol (7.0 ml/kg p.o.), isopropanol (2.5 ml/kg p.o.), and ethanol (6.0 ml/kg p.o.). Assessment was made 24 hr after the indicated doses of CCl_4.

: of the three alcohols alone did not lasma ALT activity. Ethanol pretreat-oduced an increase of ALT with the dose of CCl_4, but this was not statis-ignificant. In contrast to ethanol, both ol and isopropanol markedly poten-he CCl_4-induced increase in plasma iignificant increases were observed Cl_4 doses as low as 0.03 ml/kg with ohols. This marked potentiation by thanol and isopropanol was further trated when two of four animals pre-with either alcohol died as a result of quent 1.0 ml/kg dose of CCl_4 while ol rats tested at this dose survived. At s of CCl_4 used, methanol and isopro-otentiated the increase in plasma ALT ilar extent.

niddle panel of Fig. 2 shows the re- a similar study using hepatic accu-n of triglycerides as an index of tox-ontrol rats (those rats not pretreated ohols) showed increasing triglyceride ith increasing doses of CCl_4; however, ease was significant only at the high-of CCl_4 used. Ethanol did not signif-potentiate the CCl_4-induced increase ic triglycerides. Isopropanol pretreat-bstantially potentiated CCl_4 hepato-with doses of CCl_4 as low as 0.03 ml/ reatment with methanol also poten-CCl_4 hepatotoxicity as indicated by ng the levels of hepatic triglycerides oximately the same extent as did iso-ol. However, methanol itself produced icant increase in hepatic triglycerides isopropanol did not (Fig. 2).

effect of pretreatment with these al-on the CCl_4 dose related decrease in glucose-6-phosphatase activity (G6P) n in the bottom panel of Fig. 2. CCl_4 ontrol) at the two higher doses (0.10 0 ml/kg) significantly reduced G6P . Ethanol tended to potentiate this ut it was not statistically significant. ethanol and isopropanol potentiated ease in G6P and the degree of poten-once again appears to be similar. The ition with isopropanol was statisti-gnificant with CCl_4 doses as low as /kg. Methanol administration alone a significant decrease in G6P activity isopropanol did not.

To determine whether the hepatotoxicity of methanol (reduced G6P activity and increased triglycerides) was present at the time of CCl_4 administration, the three parameters just discussed were measured in rats 24 hr after they received methanol. The following results were obtained: Plasma ALT activity was 36 ± 6 units/ml; hepatic triglyceride levels were 5.6 ± 0.1 mg/g liver; and hepatic glucose-6-phosphatase activity was 648 ± 58 μg P/min/g liver. These values are the same as control values.

The effect of methanol pretreatment on CCl_4-induced lipid peroxidation *in vivo* is shown in Table I. Pretreatment with methanol, treatment with CCl_4 (0.10 ml/kg), or both methanol pretreatment and CCl_4 (0.10 ml/kg) did not produce a significant increase in the amount of conjugated dienes. Only injection of CCl_4 at 10 times the previously used dose (1.0 ml/kg) produced an increase in the presence of conjugated dienes. Thus, methanol pretreatment did not significantly potentiate the amount of CCl_4-induced lipid peroxidation at doses that markedly potentiated the toxicity.

The effect of pretreatment with methanol on the amount of ^{14}C irreversibly bound to microsomal protein and lipid after incubation with $^{14}CCl_4$ is shown in Table II. Pretreatment with methanol significantly increased the amount of binding of ^{14}C to both microsomal protein and microsomal lipid.

Discussion. The ability of methanol to potentiate CCl_4-induced hepatotoxicity in the rat has been established in this study. These findings confirm similar findings in mice (5). However, quantitative considerations

TABLE I. EFFECT OF METHANOL ON CCl_4-INDUCED LIPID PEROXIDATION

Treatment[a]	Conjugated dienes[b]
Saline–Corn oil	0.213 ± 0.028
Methanol–Corn oil	0.235 ± 0.048
Saline–CCl_4 (0.10 ml/kg)	0.258 ± 0.052
Methanol–CCl_4 (0.10 ml/kg)	0.273 ± 0.016
Saline–CCl_4 (1.0 ml/kg)	0.365 ± 0.041*

[a] Six rats were given saline or methanol (7.0 ml/kg) 24 hr prior to CCl_4 (i.p.) or corn oil.

[b] Expressed as OD units (243 λ) ± SE of a lipid extract obtained from microsomes from 1 g of liver 30 min after CCl_4 or corn oil injection.

* Significantly different from saline–corn oil ($P < 0.05$).

TABLE II. EFFECT OF METHANOL ON THE IRREVERSIBLE BINDING OF $^{14}CCl_4$ TO MICROSOMAL PROTEIN AND LIPID *IN VITRO*[a]

Pretreatment	nmole CCl_4 bound to protein / microsomal protein (mg)	nmole CCl_4 bound to lipid / microsomal protein (mg)
Saline	0.33 ± 0.07	2.36 ± 0.65
Methanol	0.57 ± 0.06[b]	4.08 ± 0.69[b]

[a] Microsomes were obtained from rats pretreated with either saline or methanol 24 hr earlier as described in the methods section. Values represent mean ± SE.

[b] Significantly different from saline pretreatment ($P < 0.05$).

strongly suggests a significant species difference in susceptibility to potentiating agents. Mice were found to be clearly more sensitive to isopropanol than to methanol. The present study demonstrates that the rat is equally sensitive to methanol and isopropanol as potentiators of CCl_4 hepatotoxicity. Studies have shown that the potentiation of CCl_4 hepatoxicity by isopropanol is caused by acetone, a metabolite. Whether methanol or a metabolite of methanol is responsible for the potentiation is uncertain. It is unlikely that the potentiation by methanol is due to a decrease in food consumption because even 48-hr fasting only slightly enhances CCl_4 hepatotoxicity (22).

Ethanol has been shown to potentiate CCl_4 hepatotoxicity in both rats and mice (5, 17). In the present study ethanol did not significantly potentiate CCl_4 hepatotoxicity. One possible reason for the discrepancy is that in our study a standard time interval of 24 hr between alcohol and CCl_4 was used and the time course for ethanol potentiation performed by Traiger and Plaa (5) showed that ethanol significantly potentiated only when given less than 24 hr before CCl_4. Additionally, Maling *et al.* (22) showed that repeated administration of ethanol was required to produce maximum effect.

Methanol treatment alone caused significant alteration in hepatic triglyceride levels and glucose-6-phosphatase activity but not plasma ALT activity (Fig. 2). This suggests that methanol itself has some deleterious effects on the liver. Thus, one might suspect that the enhancement of CCl_4 hepatotoxicity is merely due to the additive effects of methanol and CCl_4 on the liver. However, this is unlikely since the effect of methanol plus CCl_4 on plasma ALT is greater than additive, indicative of a potentiation phenomenon (Fig. 1).

The mechanism of CCl_4 hepatotoxicity involves the formation of a reactive metabolite, probably trichloromethyl free radical, which is capable of covalently binding to cellular macromolecules (18) and reacting with polyenoic fatty acids causing lipid peroxidation (19). Sipes *et al.* (13), using various halogenated methane derivatives, found that the potency of the hepatotoxin was directly related to the degree of irreversible binding to microsomal protein. Pretreatment of rats with isopropanol was found to increase the irreversible binding of CCl_4 to liver lipids and proteins *in vivo* (20) and *in vitro* (21). In the present study methanol pretreatment significantly increased the amount of ^{14}C irreversibly bound *in vitro* after incubation with $^{14}CCl_4$. This suggests that the two alcohols are potentiating the hepatotoxicity by increasing the irreversible binding of CCl_4. An increase in CCl_4 covalent binding may be the result of an increased metabolism of CCl_4. Powis (7) has demonstrated that methanol, isopropanol, and ethanol can cause alterations in hepatic microsomal metabolism of foreign compounds in the rat.

Methanol pretreatment did not significantly increase CCl_4-induced lipid peroxidation. Similar studies with isopropanol produced conflicting results. Maling *et al.* (22) showed that pretreatment with isopropanol increased CCl_4-induced lipid peroxidation. Lindstrom and Anders (23) showed that isopropanol pretreatment produced only a transient increase in CCl_4-induced lipid peroxidation. They, therefore, concluded that lipid peroxidation may not be associated with the increase in CCl_4 toxicity observed after acute administration of ethanol or isopropanol. Results from the present study would agree in that it appears that covalent binding is more closely associated with the increased CCl_4 hepatotoxicity caused by methanol than is lipid peroxidation.

Summary. The potentiation of CCl_4-induced hepatotoxicity resulting from oral administration of methanol has been investigated in the rat. Results from the study demonstrate that methanol potentiates CCl_4

hepatotoxicity to the same extent as that observed with isopropanol. It appears that the mechanism by which these two alcohols potentiate is similar in that they both increase covalent binding of CCl_4 to microsomal proteins and lipids. However, the precise mechanism of potentiation remains as yet undetermined.

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Prostacyclin (PGI_2) Elicits Reflex Bradycardia in Dogs: Evidence for Vagal Mediation (40625)

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Prostacyclin (PGI_2), the recently discovered naturally occurring product of arachidonic acid (AA) metabolism when injected into the dog causes a reduction in both heart rate and blood pressure (1). Since other prostaglandins (PG) have been shown to stimulate cardiovascular afferent neurons in the lung and heart (2) and since reflex reductions in heart rate occur following the injection of veratrum alkaloids (3), bradykinin (4), and episodes of myocardial ischemia (5), we speculated that the bradycardia induced by PGI_2 might also be reflex in origin. The current study describes the cardiovascular effects of PGI_2 and provides evidence that the PGI_2-induced bradycardia is a reflex that originates with the stimulation of cardiopulmonary receptors and is mediated by vagal nerve fibers.

Methods. Preparation of animals. Thirty-three male mongrel dogs (17–35 kg) were anesthetized with a mixture of equal amounts of Dial-urethane (Ciba–Geigy) and Nembutal (Abbott). Either the thorax was opened in the fourth left intercostal space for the placement of electromagnetic flow probes (Carolina Medical) and pressure cannulas in open-chest dogs as previously reported (6) or catheters and a catheter-tippped flow probe were advanced into the left ventricle and aorta for the measurement of left ventricular pressure and cardiac output, and into the pulmonary artery for pressure recording and the injection of drugs in closed-chest animals. Systemic blood pressure was measured from a cannula in the brachial artery, and heart rate was calculated from the pressure pulse interval electronically (Narco). Maximum *dP/dt* was derived from the ventricular pressure recording and used as an indication of cardiac contractility. All data were recorded on a direct-writing oscillograph (Physiograph 6B, Narco). The animals were intubated and artificially respirated (Harvard Instruments Co.), warmed with a water-circulating heating system (Thermo-rite), and allowed at least 30 min to stabilize before injections of various agents.

Preparation of drugs. Stock solutions of PGI_2 were prepared fresh every day in 1 *M* Tris buffer (pH 9.3) at a concentration of 1 mg/ml and diluted just prior to injection in 50 m*M* Tris (pH 7.8). Arachidonic acid (Nu-chek) and PGs were prepared as sodium salts at concentrations of 1 mg/ml and diluted just prior to use in normal saline. Injections of vehicles were without effect.

Statistical analysis was performed using Student's *t* test for paired values (7).

Results. Intravenous administration of PGI_2 (1–30 μg) caused a fall in blood pressure and heart rate accompanied by a reduction in left ventricular systolic pressure, pulmonary artery pressure, and maximum *dP/dt*, together with an increase in cardiac output (Fig. 1). In 9 of 13 closed-chest and 15 of 20 open-chest dogs PGI_2 induced a fall in heart rate from 170 ± 5 to 151 ± 6 (mean ± SE) and 162 ± 5 to 144 ± 5 beats/min, respectively (Table I). Additionally, in open-chest dogs, injection of PGI_2 caused a 103% increase in stroke volume (10.2 to 20.7 nl) with a 9% increase in stroke work (1700 to 1950 g·cm/stroke). There was a 69% reduction in total peripheral resistance (5924 to 1875 dyne·sec/cm^5) and a 66% reduction in pulmonary vascular resistance (982 to 431 dyne·sec/cm^5). The 75% increase in cardiac output (1650 to 2890 ml/min) was achieved at a 3% reduction in cardiac work (2.75 to 2.67 kg·m/min), indicating an improved cardiac efficiency. (Above values derived from Table I.)

Increasing the dose of PGI_2 did not consistently increase the maximum fall in heart rate; however, there was a threshold dose of PGI_2 that differed from dog to dog. Also, once a response was elicited in a dog, it was reproducible throughout the experiment. In

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ıe dogs that did not exhibit the bradycardia
› PGI_2, there was either no increase in heart
ıte or the increase was less than that seen to

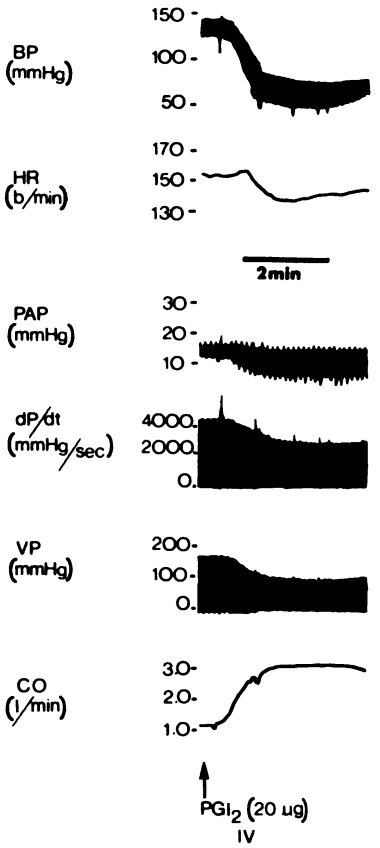

FIG. 1. The intravenous injection of PGI_2 (20 μg) ›o the anesthetized open-chest dog caused a fall in ›od pressure (BP), heart rate (HR), pulmonary artery ɛssure (PAP), maximum *dP/dt*, ventricular pressure P), and an increase in cardiac output (CO).

a comparable fall in blood pressure caused by nitroprusside or PGE_2. Injection of PGE_2 and PGE_1 (50 μg), both potent vasodepressors in the dog, induced as expected a reduction in blood pressure that was closely followed by a baroreceptor-mediated increase in heart rate (8). Nitroprusside (500 μg), a drug whose systemic and pulmonary vascular activity is similar to PGI_2 (9), was administered into the pulmonary artery of dogs to establish whether dilation of pulmonary blood vessels per se might be responsible for a fall in heart rate. As with PGE_2, injection of nitroprusside always resulted in a fall in left ventricular and systemic blood pressures, followed by increases in heart rate and in maximum *dP/dt*, a pattern of responses characteristic of the effects on baroreceptors of lowering blood pressure in anesthetized dogs (Fig. 2).

Administration of PGI_2 into the pulmonary artery (Fig. 2), the brachial or femoral veins, the left circumflex coronary artery, and the left atrium or the left ventricle, all elicited bradycardia despite the acute and profound reduction in blood pressure in both open- and closed-chest anesthetized dogs. On the other hand, a baroreceptor-induced tachycardia resulted from the injection of equivalent amounts of PGI_2 into the carotid artery and the ascending or descending aorta (13 ± 3.1 beats/min and 22.6 ± 6.5 beats/min increase in the open- and closed-chest dogs, respectively) (Fig. 3) suggesting that the bradycardia is not brought about by a direct effect on peripheral baroreceptors or chemoreceptors but on an as yet undefined population of cardiopulmonary receptors. Arachidonic acid (1–5 mg), the fatty acid precursor of the 2-

TABLE I. HEMODYNAMIC CHANGES IN OPEN-CHEST DOGS INDUCED BY PGI_2[a]

	Heart rate (beats/min)	Mean arterial blood pressure (mm Hg)	Cardiac output (liters/min)	Mean pulmonary artery pressure (mm Hg)	*dP/dt* (mm Hg/sec)	Systolic ventricular pressure (mm Hg)
›ontrol	162.3 ± 5.5 (15)	122.5 ± 5.5 (15)	1.65 ± 0.29 (13)	20.3 ± 1.6 (15)	4986 ± 330 (7)	139.0 ± 8.5 (7)
›GI₂	144.4 ± 5.3 (15)	67.9 ± 4.0 (15)	2.89 ± 0.41 (13)	15.6 ± 1.4 (15)	3686 ± 233 (7)	93.0 ± 6.9 (7)
›ercentage change	−11	−45	+75	−23	−26	−33

[a] Depicted are the peak changes caused by the lowest effective dose of PGI_2 for reduction of heart rate in each g. Values shown are mean ± SE. Number of dogs in parentheses. All changes following injection of PGI_2 are nificantly different from control ($P < 0.0001$).

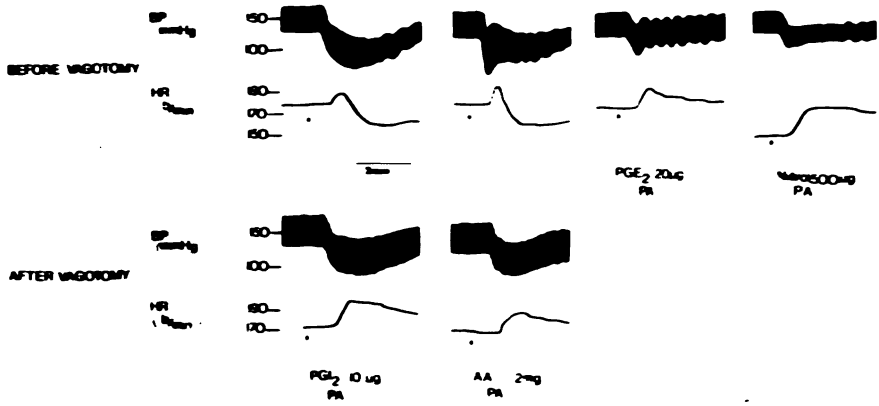

FIG. 2. Injection of PGI$_2$ or arachidonic acid (AA) into the pulmonary artery (PA) of a dog caused a fall in blood pressure concomitant with a reduction in heart rate. The transient increase in heart rate that precedes the bradycardia is only occasionally observed. PGE$_2$ and nitroprusside (Nitro) reduce pressure and increase heart rate. Vagotomy abolishes the fall in heart rate to PGI$_2$ and AA. On the other hand, vagotomy leaves the heart rate response both to PGE$_2$ and nitroprusside unchanged (not shown). The above records are from a single dog.

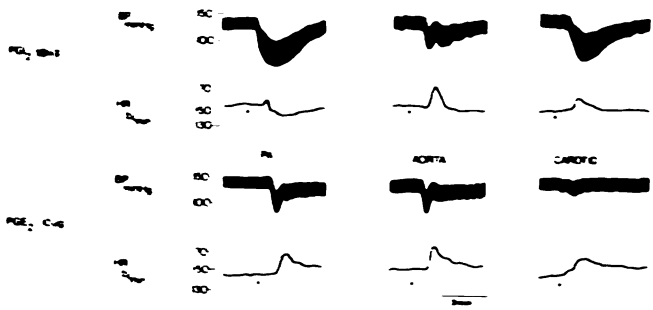

FIG. 3. Injection of PGI$_2$ into the pulmonary artery (PA) causes hypotension and bradycardia. Injections into the aorta and carotid artery result in tachycardia and a blood pressure fall which is significantly less than that seen with PA injection. Regardless of the site of the injection PGE$_2$ always induces a reduction in blood pressure that is accompanied by an increase in heart rate. The above record is from a single, closed-chest dog.

series prostaglandins, when injected into dogs not only caused a reduction in blood pressure due to the production of PGs (10) but also induced a bradycardia similar to that caused by PGI$_2$ (Fig. 2). Moreover, 6-oxo-PGF$_{1\alpha}$ (100–500 µg), the major metabolite of PGI$_2$, had no effect on blood pressure or heart rate; hence, the bradycardia is most likely due to PGI$_2$ and not to its breakdown products.

Bilateral cervical vagal section (Fig. 2) or the administration of atropine (1 mg/kg) eliminated the cardiac slowing that follows the injection of PGI$_2$ or arachidonic acid (11, 12) and allowed the typical baroreceptor-mediated increase in heart rate, that follows the lowering of blood pressure, to appear. These results confirm that the bradycardia is not due to a direct effect of PGI$_2$ on cardiac pacemaker cells but rather is reflexly mediated by vagal fibers. Moreover, vagotomy (Fig. 2) or intraaortic injection (Fig. 3) attenuated the mean blood pressure fall to prostacyclin (50.6 ± 3.8 to 37.0 ± 3.0 and to 28.6 ± 1.4 mm Hg, respectively) indicating that in addition to the direct vasodilator activity a significant portion of the vasodepressor effect of PGI$_2$ is due to a reflex reduction in peripheral resistance that is perhaps partially attributable to an inhibition of the baroreceptor-mediated increase in peripheral vascular tone.

Discussion. Prostacyclin like other vasodilator prostaglandins reduces blood pressure (13), but unlike PGE$_1$ or PGE$_2$ it also lowers significantly pulmonary artery pressure (9). These actions depend primarily on the direct

of PGI_2 on vascular smooth muscle
ıt also, as our experiments suggest, on
x reduction of peripheral resistance.
ıdden decrease in afterload leads to
ventricular systolic pressure and car-
ntractility (max dP/dt), and also to an
e in venous return. Thus, in spite of a
on in heart rate, cardiac output, due
:nlarged stroke volume, increases sig-
tly.

ɔprusside is a clinically useful agent,
t decreases pressure in both the pul-
y and systemic circulations and con-
tly affects the pressure–volume inter-
ıship of the ventricles by reducing af-
(15). Prostacyclin has vasodepressor
r similar to nitroprusside (9) thus de-
g cardiac work; additionally, since
ɛduces heart rate, it may further lower
rdial O_2 consumption.

cardiovascular profile of PGI_2, espe-
he effects on blood pressure and heart
similar to that of veratrine as initially
:d by von Bezold and Hirt (16) and
ɔnfirmed by Jarisch and Richter (17).
:ceptors responsible for the "Bezold–
" effect have been localized to the left
le (18) and the coronary circulation
larly the area supplied by the left cir-
x coronary artery (3, 5). Stimulation of
'eceptors leads to an increase in the
ɔf vagal c-fibers (19) and a reduction
t rate which is abolished by vagotomy
he physiologic function of these recep-
ay be to prevent ventricular overload
nd, by reducing both afterload and
'ate, to decrease cardiac work during
rdial ischemia (21).

ıs recently been established that PGI_2,
:ed by vascular endothelial cells, is a
antiplatelet aggregatory substance
n addition to its dilator effect in the
ry vascular bed (23), infusion of PGI_2
e cat following coronary ligation has
ıown to stabilize lysosomes and aid in
ening of collaterals and thus protect
myocardial ischemic injury (24). Fi-
ɛince prostaglandin production under
l physiologic conditions is low, the re-
ɪduction of heart rate we observed may
ɔnly limited import; however, since
ɛ probably a circulating hormone (25)
nce during injury due to ischemia, trauma, or other types of tissue injury, prostaglandin production is enhanced (26), this reflex may be of primary pathophysiologic importance. Additionally, it may serve to explain the bradycardia observed during cardiogenic shock (27), coronary arteriography (28), administration of acetylstrophanthidin (29), posterior infarction (30), and myocardial ischemia (31).

Summary. Administration of the vasodepressor prostaglandins, PGE_1 and PGE_2, and of nitroprusside, an agent whose direct vascular activity is similar to prostaglandins, resulted in a reduction in systemic arterial blood pressure accompanied by the expected reflex increase in heart rate. Injection of prostacyclin (PGI_2) and the prostaglandin precursor, arachidonic acid, into the femoral vein, pulmonary artery, left atrium, and left ventricle of the dog elicited a fall in blood pressure and a concomitant reduction in heart rate in both open- and closed-chest anesthetized dogs. Bilateral vagal section eliminated the bradycardia thus establishing that the PGI_2-induced change in heart rate is reflex in origin.

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l Pressure in Atherosclerosis-Susceptible and -Resistant Pigeons (40626)

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Carneau (WC) pigeons in contrast Racer (SR) pigeons at a similar olesterol concentration have aortic rosis that develops naturally even animals are fed cholesterol-free s (1, 2). It has been suggested by ors (3) that blood pressure increases it not in SR pigeons from 9 to 17 age may be related to the increased rosis of the WC pigeon since it is at he birds begin to develop aortic n an attempt to determine a rela- etween blood pressure and aortic rosis, Middleton *et al.* (4) studied r-old WC pigeons and concluded elationship existed. Because of the of the WC pigeon in atheroscle- rch, we designed a study to inves- ors effecting blood pressure in this compared these findings with the rosis-resistant SR pigeon. In this provide information to determine ial, age, and sex influence on blood s well as the relationship of blood nd diet-induced atherosclerosis of ns.

ls and methods. The pigeons used ly were obtained from the breeding t the Bowman Gray School of Med- rch farm. WC pigeons were derived lony of pigeons that were mated randomly; SR pigeons were from 37 and SR 39 (5) maintained and omly for at least 16 generations. All ere housed in outdoor flypens as by Clarkson *et al.* (6) and subjected nmental conditions prevailing in alem (described in detail by Nohl-Wagner (7)).

ressure measurement. Blood pres- measured directly from the alar restrained, unanesthetized, hooded physiograph 4 and Model P1000 ransducer (E & M Instrument Co., ton, Tex.) were used initially. Later studies were done using a Grass Model 5 physiograph (Grass Instrument Co., Quincy, Mass.). The needle of a 23-gauge butterfly infusion set was filled with heparinized saline and inserted into the right alar artery. Mean blood pressures were determined by dampening the pressure transmitted to the transducer in a gradual and complete manner by occluding the cannula with a hemostat. Throughout the study we routinely obtained a continuous tracing of blood pressure for 5 min postcannulation and usually selected the 3-min values to represent the basal blood pressure for an animal.

Seasonal effect on blood pressure. Seasonal influence on blood pressure was determined from monthly observations on 13 WC pigeons ranging in age from 3 to 9 years.

Effect of age, sex, and breed on blood pressure. Eighty WC, 50 SR 37, and 21 SR 39 distributed nearly equally among the ages of 3–5 months, 1, 3, 4, 6, or 8 years were used to evaluate any effects of age, sex, or breed on blood pressure. Housing was as follows: flypen 1, 3–5 month and 8-year-old pigeons; flypen 2, 3- and 6-year-old pigeons; flypen 3, 1- and 4-year-old pigeons. All blood pressure measurements were done during a 19-day period on WC and SR pigeons chosen randomly from each of the pens of animals. Blood pressure was determined on the basis of a single observation for each animal. These measurements were repeated on all animals 6 months later (October) and found to differ by only 6.6, 7.2, and 10.2% for mean, diastolic, and systolic pressures, respectively, for both WC and SR pigeons.

Relationship of blood pressure and aortic atherosclerosis. The association of blood pressure and atherosclerosis was evaluated in 184 WC pigeons of either sex and 18–22 weeks of age. The blood pressure was measured for each animal and then all pigeons were fed an atherogenic diet (Purina pigeon pellets:lard: cholesterol; 89.75/10.0/0.25) for 6 months.

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Blood pressure was determined at the end of 6 months and the animals were killed. The extent and severity of aortic atherosclerosis was determined as described previously (8).

Statistical analysis of data. Statistical significance of data was determined by one or more of the following statistical tests: paired or nonpaired Student's *t* test, analysis of variance, and Duncan's multiple range test or Tukey's HSD.

Results and discussion. Preliminary studies using three WC pigeons indicated very little fluctuation in mean blood pressure from 1 through 30 min postcannulation. The measurements taken during the first several minutes postcannulation appeared to be representative of a basal blood pressure. When 13 WC pigeons were studied, the group had an average mean blood pressure of 155–159 mm Hg with standard deviations of only 3–5 at minute intervals from 1 to 6 min postcannulation.

The variability in blood pressure within and between WC pigeons during a 1-year period is illustrated in Fig. 1. Ranges of 182–194 and 128–141 mm Hg were seen, respectively, for systolic and diastolic measurements. The data in Fig. 1 indicate that the systolic, mean, and diastolic blood pressures showed no consistent fluctuations attributable to the time of the year that the blood pressures were measured. When the blood pressure values of all pigeons were averaged no significant influence of season was seen. However, an impression of lower systolic pressures in the summer months and higher systolic pressures in winter months was obtained. An influence of a seasonal effect on blood pressure has been reported in chickens by Weiss *et al.* (9). Higher systolic pressures in winter and lower pressures in summer months were reported for 219 adult chickens. These seasonal differences were reproducible over several seasonal cycles and approximated a 10% decrease from winter to summer for both sexes. Weiss *et al.* (9) indicated that the lower systolic blood pressures in chickens in warm weather were the results of the ambient temperature and not the amount of light.

It is interesting that even under the influence of our moderate climate, we saw a trend of a seasonal effect. Mean monthly temperatures from June to September when the study was done ranged from 21 to 27°. Apparently cardiovascular mechanisms resulting in reduced blood pressure operate at these temperatures. The effect on blood pressure may result in part from a reduced total peripheral resistance through vasodilation to provide for body temperature regulation through evaporative cooling by panting.

The effect of age and sex on systolic and diastolic blood pressure in WC and SR pigeons is illustrated by Fig. 2. There were no differences in blood pressure of SR 37 and SR 39 pigeons and the data for these two lines of SR pigeons were combined. For WC pigeons, the results of an analysis of variance indicated no significant effect of age on blood pressure. In SR pigeons, no major differences were observed in blood pressure throughout the age range studied. Young SR pigeons appeared to have a higher diastolic pressure than older pigeons. Mean values for 1-year-old pigeons were significantly ($P < 0.05$)

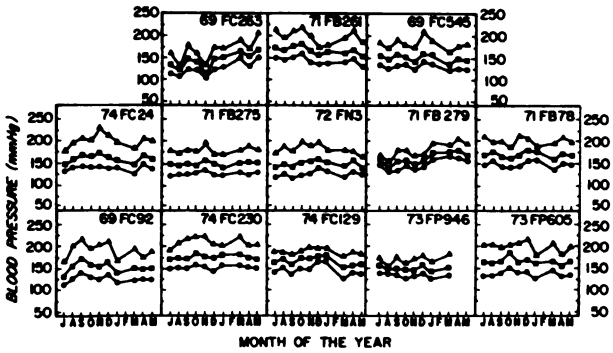
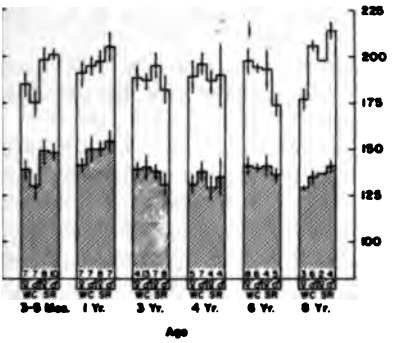


FIG. 1. Systolic (▲), mean (■), and diastolic (●) blood pressures of White Carneau pigeons during a 1-year period. Letters indicate month of the year.

ıan those of 3, 4, and 5 years of age
o 5-month-old pigeons were signifi-
igher than those of 3 and 4 years of

been reported that chickens have a
nt increase in blood pressure from
14 months to ages 42–54 months (10).
tion on the WC pigeon (3) indicated
systolic pressure increased from 155
at 9 months of age to 169 mm/Hg at
ths of age but SR pigeons did not
y age-related increase. In this study,
did we see significantly higher blood
values in WC compared with SR
Additionally, our results indicate no
e of the sex of the pigeon on blood
of either WC or SR pigeons.
ndings that blood pressure increases
ıpparent in WC pigeons from 6 to 12
of age when naturally occurring ath-



The effect of age and sex on blood pressure
Carneau (WC) and Show Racer (SR) pigeons.
cross-hatched portion of each bar represents
liastolic blood pressure. Upper portion of each
ents the mean systolic blood pressure. Vertical
ate ± SEM. Numbers at the bottom of each
te the number of studied. Significant differ-
0.05, SR 3–5 months > SR 3 and 4 years; SR
R 3, 4, and 5 years.

erosclerosis begins and that WC and SR pigeons have similar blood pressure levels indicate that blood pressure increases do not account for the atherosclerosis susceptibility characteristic of the WC pigeon.

WC pigeons fed an atherogenic diet for 6 months had 4.7 ± 0.19% (mean ± SEM) of the aortic surface covered with raised atherosclerotic plaque. The values for extensiveness of atherosclerosis ranged from 0 to 38% in the group. On the basis of studies of the natural progression of atherosclerosis in the WC pigeon (1) it is probable that no or very little atherosclerosis was present in the 18- to 22-week-old pigeons at the beginning of the study and the aortic plaques were in large part induced by diet. As an estimate of the severity of the diet-induced atherosclerosis, aortic cholesterol concentration, and content were, respectively, 4.48 ± 0.19 mg/g wet aorta and 0.409 ± 0.07 mg/aorta/500 g body wt (mean ± SEM). For comparison, those pigeons without visible atherosclerosis had aortic cholesterol concentrations of 1.36 mg/g and contents of 0.124 mg/aorta/500 g body wt ($n = 28$).

In the entire population of pigeons studied, there were small but significant ($P < 0.01$) increases in mean and diastolic blood presures after the 6-month period that the atherogenic diet was fed (Table I). In an attempt to more clearly evaluate blood pressure changes in pigeons with and without appreciable atherosclerosis, the entire group was subdivided into animals having aortic cholesterol contents either no greater than 0.15 mg or greater than 0.50 mg. These groups represented animals with minimal or no atherosclerosis (1.2% of the aortic surface with plaque) and animals with extensive atherosclerosis (16% of the aortic surface with

.E I. Blood Pressure of White Carneau Pigeons Fed an Atherogenic Diet for 6 Months

	mg chol/ aorta/500 g body wt	% Intimal surface with raised atherosclerotic plaque	N	Preexperimental blood pressure (mm Hg) Systolic	Mean	Diastolic	6-Month blood pressure (mm Hg) Systolic	Mean	Diastolic
	0.409 ± 0.837	4.7 ± 6.7	184	183 ± 13	154 ± 11	137 ± 11	185 ± 18	158 ± 14*	141 ± 13*
nin	0.129 ± 0.018 (≤0.15)	1.2 ± 2.5	44	182 ± 13	152 ± 11	135 ± 9	177 ± 17	154 ± 9	137 ± 11
nin	1.504 ± 1.860 (≥0.50)	16.0 ± 10.3	23	182 ± 11	153 ± 13	137 ± 9	195 ± 17*,†	164 ± 15*,†	146 ± 13*,†

are means ± SD.
nt differences with groups, $P < 0.01$.
nt differences between groups with minimum and extensive atherosclerosis, $P < 0.01$.

plaque) (Table I).

Pigeons with extensive atherosclerosis had significantly greater systolic, diastolic, and mean blood pressures in comparison to pigeons with little or no atherosclerosis. Pressures after 6 months were elevated significantly above the preexperimental values only in animals with extensive and severe atherosclerosis. No blood pressure differences were seen in any of the three groups of pigeons at the beginning of the study (Table I). Since the pressure changes were seen only after pigeons had extensive atherosclerosis and were not present in animals with minimal levels of atherosclerosis, the data support the contention that blood pressure increases in WC pigeons are a result of atherosclerosis. Dietary aggravated plaques are more severe than those in naturally occurring atherosclerosis and it is reasonable to assume that this accounts for our findings on the lack of increase of blood pressure with increasing age in the WC pigeon.

Summary. Studies were designed to determine the influence of season, age, sex, and atherosclerosis on blood pressure in the White Carneau (WC) pigeon, a breed that develops naturally occurring atherosclerosis. Comparisons were made with the Show Racer (SR) pigeon, a breed that is resistant to aortic atherosclerosis. In WC pigeons of 3–5 months, 1, 3, 4, 5, or 8 years of age, no influence of age or sex of the pigeon on blood pressure was measured. At no age did we see significantly higher blood pressure levels in WC compared with SR pigeons. WC pigeons, of an age prior to the development of naturally occurring atherosclerosis, were fed an atherogenic diet for 6 months. Only pigeons with extensive atherosclerosis had significantly greater systolic, diastolic, and mean blood pressures. The study provides evidence that blood pressure increases do not account for the atherosclerosis susceptibility of WC pigeons and that extensive and severe atherosclerosis result in elevated blood pressure levels in WC pigeons.

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ıction of Adrenergic Stimuli, Prostaglandins and Angiotensin II in the Dog Kidney (40627)

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ıs been previously shown that intraar-
nfusion of angiotensin II (AII) poten-
the renal and mesenteric vascular re-
to adrenergic stimuli (1, 2). The cuta-
vascular response to sympathetic nerve
ation was potentiated to a greater de-
ıan the response to exogenous norepi-
ne (NE) (3). The exact mechanism by
this potentiation occurs remains to be
:d. Previous reports have suggested that
ıcilitates NE release from adrenergic
terminals (4), enhances NE biosyn-
(5), and inhibits neuronal uptake of
). Malik and Nasjletti (7) have impli-
AII receptors in this process by dem-
ting that prior treatment with the an-
sin II antagonist, 1-sarcosine-8-isoleu-
ıgiotensin II (AIIA), abolished the po-
ion by AII of mesenteric vascular re-
s to adrenergic stimuli.

ıough the potentiation by exogenous
renal vascular responses to adrenergic
i has been clearly demonstrated, the
logical role of this phenomenon is still
stion. One purpose of this study was to
ıine if endogenous circulating levels of
resent in the anesthetized dog, poten-
ıe renal vascular response to exogenous
renal nerve stimulation (RNS). It was
ed that if endogenous circulating levels
do potentiate renal vascular responses
energic stimuli then the magnitude of
esponses should be decreased by renal
de to circulating AII.

ıtaglandins (PG) have also been impli-
as agents which modify the responses
:nergic stimuli in various vascular beds
. The responses, however, are species
dent and differences have been noted
:n the rat and rabbit renal vasculature
:ostaglandins, when infused into the
ırtery, are capable of selectively mod-
; responses to adrenergic stimuli (11–
the dog (11), rabbit (12), and cat (13)
kidney, intrarenal infusion of PGE_2 abolished the renal vasoconstrictor response to RNS but had little effect on the renal vasoconstrictor response to exogenous NE.

Indomethacin and meclofenemate, inhibitors of PG synthesis, have been utilized to assess the role of endogenous PG in this process. In the isolated-perfused rabbit heart indomethacin augmented NE release after sympathetic nerve stimulation (14). Zimmerman and colleagues (8) reported that, in the perfused dog paw, indomethacin potentiated the cutaneous vascular response to both NE and nerve stimulation. Chapnick and colleagues (15) demonstrated that, in the cat kidney, indomethacin enhanced the renal vascular response to exogenous NE but not RNS whereas meclofenemate had no effect on the renal vascular response to either exogenous NE or RNS.

The second purpose of this study was to evaluate the role of endogenous PG in modifying the vascular responses to RNS and exogenous NE in the canine renal vascular bed.

In anesthetized dogs systemic indomethacin administration increases mean arterial pressure (MAP), reduces renal blood flow (RBF), and increases renal vascular resistance (RVR) (16). A study of Mimran and colleagues (17) in the rat implicated the renin–angiotensin system in this process. Bilateral nephrectomy abolished the rise in MAP normally seen with indomethacin treatment and in normal rats systemic administration of AIIA prevented the indomethacin-induced increase in MAP and decreases in RBF.

The third purpose of this study was to examine the possible involvement of the renin–angiotensin and adrenergic nervous systems in the renal vasoconstrictor response to systemic administration of indomethacin and meclofenemate in the dog.

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Materials and methods. All studies were performed on female mongrel dogs, 15–20 kg in weight, fed a standard kennel ration. On the day prior to the study all dogs were deprived of food but water was permitted *ad libitum*. On the day of the study the animal was anesthetized with i.v. sodium pentobarbital 30 mg/kg and supplemental doses were added throughout the experiment to maintain anesthesia. The animal was intubated with an endotracheal tube and mechanically ventilated to maintain arterial pH between 7.35 and 7.45.

Catheters were inserted into the right femoral artery, inferior vena cava via right femoral vein, and left jugular vein to permit sampling of arterial blood, arterial blood pressure measurements, and intravenous infusion of fluids. The left kidney was exposed via a left subcostal incision, cleared of perirenal tissue, and supported in a Lucite cup ring. The renal nerves were dissected free and platinum electrodes were placed on the transected distal portion. Small catheters were placed in the left and right ureters. An external flow probe was placed on the left renal artery and led to an electromagnetic flow meter (Carolina); this system was calibrated *in situ* and *in vivo* at the end of each experiment. For one series of experiments an additional external flow probe was placed on the right renal artery. A 25-gauge curved needle attached to polyethylene tubing was placed in the left renal artery against the direction of flow. The tubing was connected to an infusion pump and 0.9% NaCl was infused at 0.5 ml/min. This procedure did not affect the signal output from the external flow probe.

At the conclusion of surgery a priming dose of inulin was given followed by its constant infusion in 0.9% NaCl at 1.0 ml/min to maintain a plasma concentration of 0.02 mg/ml. At least 60 min were allowed for equilibration following surgery.

Plasma and urine samples were analyzed for inulin by the anthrone method (18). The clearance of inulin was taken to be glomerular filtration rate (GFR). MAP was measured with a pressure transducer. RBF and MAP were recorded on a direct writing recorder. RVR (mm Hg/ml/min) was calculated by dividing MAP by RBF.

In one group of studies, left kidney AII receptor blockade was achieved by the intrarenal arterial infusion of 1-sarcosine-8-isoleucine angiotensin II (AIIA) at 0.2 μg/kg/min. After 20 min of AIIA infusion, the efficacy of blockage was verified by the failure to note changes in RBF after intrarenal arterial doses of AII (1–5 μg) which prior to the infusion of AIIA reduced RBF by 30–50%. As in previous studies employing this AIIA (19), intrarenal arterial infusion of AIIA alone did not affect RBF (251 ± 29 vs 249 ± 25 ml/min, $N = 8$, see Fig. 1) nor did it abolish the systemic pressor response to intravenous administration of 10 μg AII (+ 35 ± 6 vs + 37 ± 5 mm Hg, $N = 8$) or the contralateral renal vasoconstrictor response to intrarenal arterial administration of AII (−92 ± 12 vs −96 ± 13 ml/min, $N = 8$).

In separate groups of studies indomethacin or meclofenemate were used to inhibit PG synthesis. Indomethacin (2 mg/kg), dissolved by the method of Davis and Horton (20) and meclofenemate (2 mg/kg), dissolved in 0.9% NaCl, were infused intravenously over a 10-min period. Following infusion, a 20-min equilibration period preceded all tests. Studies by Kaloyanides and colleagues (21), Dunn and colleagues (22), and Zambraski and Dunn (23) indicate that these doses of indomethacin or meclofenemate reduce renal

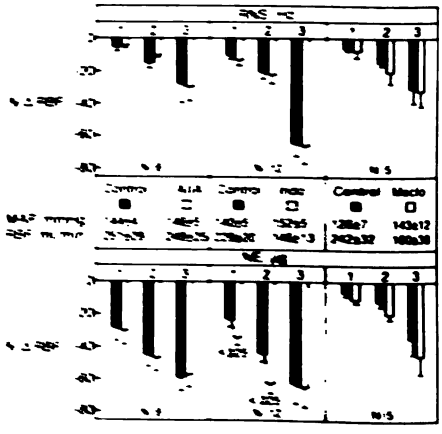

Fig. 1. Renal blood flow (RBF) responses to graded renal nerve stimulation (RNS) and doses of exogenous norepinephrine (NE) before and after intrarenal angiotensin II blockade (left panel) and systemic administration of indomethacin (middle panel) and meclofenemate (right panel).

PGE_2 secretion and excretion. In addition, the ability of indomethacin and meclofenemate to block the renal vasodilator response to arachidonic acid (dissolved in 0.1 *M* sodium carbonate in 10% ethanol) was taken as an indication of reduction of enzymatic conversion of arachidonic acid to vasodilator prostaglandins (24).

In another group of studies, renal α-adrenergic receptor blockage was produced by the intrarenal arterial infusion of phenoxybenzamine, 0.2 μg/kg/min. Adequacy of renal α-adrenergic receptor blockade was evaluated via renal intrarterial injections of phenylephrine 10–40 μg which produced 50–75% reductions in RBF in the absence of phenoxybenzamine. Previous studies (25, 26) have demonstrated that this dose of phenoxybenzamine infused into the renal artery does not result in systemic α-adrenergic receptor blockade.

Direct electrical stimulation of the renal nerves was performed using a Grass S9 stimulator at 10–20 V, and 1.0 msec. The frequency of renal nerve stimulation was varied in each animal to achieve approximately 10, 20, and 50% decreases in RBF. Each stimulation period lasted approximately 2 min, at which time RBF had decreased and remained constant. The different intensities of stimulation were performed in random order and a time interval sufficient for complete recovery, 5- to 10-min duration, was allowed between successive stimulations.

Norepinephrine was administered as norepinephrine bitartrate, dose in terms of base, in a constant volume into the renal artery infusion line. The different doses of norepinephrine were administered in random order and a time interval sufficient for complete recovery, 5- to 10-min duration, was allowed between successive doses.

Renal nerve stimulation and norepinephrine administration were done in the same animal before and after intrarenal angiotensin II blockade ($N = 8$ dogs), systemic administration of indomethacin ($N = 12$ dogs), and meclofenemate ($N = 5$ dogs).

The data in the text and figures are expressed as the mean ± standard error. A Student's *t* test (27) was used for statistical analysis of paired data within each group.

Results. The results of the studies concerning the effect of renal AII blockade and inhibition of prostaglandin synthesis on renal vascular responses to RNS and exogenous NE are shown in Fig. 1. Renal AII blockade did not significantly change baseline MAP or RBF nor did it alter the renal vascular responses to RNS or exogenous NE.

The results of the studies concerning the ability of indomethacin and meclofenemate to block the renal vasodilator response to arachidonic acid are shown in Table I. The intrarenal arterial administration of arachidonic acid produced dose-related increases in RBF. The administration of indomethacin and meclofenemate produced changes in basal MAP and RBF; indomethacin increased MAP from 142 ± 4 to 152 ± 5 mm Hg and decreased RBF from 201 ± 8 to 146 ± 23 ml/min and meclofenemate increased MAP from 121 ± 10 to 131 ± 9 mmHg and decreased RBF from 179 ± 29 to 144 ± 27 ml/min. The renal vasodilator responses to arachidonic acid were decreased by an average of 50% by both indomethacin and meclofenemate, similar to the findings of Feigen and colleagues (9).

The results of the studies concerning the effect of inhibition of prostaglandin synthesis on renal vascular responses to RNS and exogenous NE are shown in Fig. 1. Indomethacin administration increased baseline MAP by 9% ($P < 0.01$) and decreased baseline RBF by 36% ($P < 0.001$). Meclofenemate administration increased baseline MAP by 13% ($P < 0.05$) and decreased baseline RBF by 34% ($P < 0.025$). Neither indomethacin nor meclofenemate altered the renal vascular response to RNS. Indomethacin significantly increased the renal vasoconstrictor responses

TABLE I. EFFECT OF INDOMETHACIN AND MECLOFENEMATE ON RENAL VASODILATOR RESPONSES TO ARACHIDONIC ACID[a]

Dose (mg)	*N*	Control ΔRBF (ml/min)	Indomethacin ΔRBF (ml/min)	Meclofenemate ΔRBF (ml/min)
0.1	4	10 ± 3	6 ± 1	
0.3	4	25 ± 5	7 ± 1	
1.0	4	39 ± 6	15 ± 4	
0.1	4	8 ± 1		4 ± 2
0.3	4	16 ± 2		8 ± 1
1.0	4	50 ± 8		23 ± 4

[a] Data are mean ± SE; *N* = number of animals.

to the 1- and 2-μg doses of exogenous NE ($P < 0.025$) but did not influence the response to the 3-μg dose of exogenous NE. Meclofenemate did not alter the renal vascular responses to RNS or exogenous NE.

The results of studies concerning the mechanism of the renal vasoconstrictor response following indomethacin and meclofenemate administration are shown in Figs. 2–4. In Fig. 2, the renal vascular response to systemic indomethacin is monitored bilaterally; blockade to circulating AII was produced in the left kidney while the right kidney of the same dog served as an untouched internal control. Similar decreases in RBF and GFR were observed in both left and right kidneys. Figure 3 illustrates data from similarly designed experiments with meclofenemate; again, the alterations in RBF and GFR were similar in the two kidneys. These data indicate that the renal vasoconstriction observed after systemic indomethacin or meclofenemate administration is not mediated by circulating AII. In Fig. 4, both AII and α-adrenergic receptor blockade were produced in the left kidney. Systemic indomethacin administration produced an increase in MAP from 129 ± 3 to 140 ± 4 mm Hg, a decrease in RBF from 218 ± 33 to 170 ± 26 ml/min, and an increase in RVR from 0.712 ± 0.157 to 1.120 ± 0.376 mm Hg/ml/min (all $P < 0.01$). These responses are similar to those previously observed in our laboratory (28) when indomethacin was administered alone and to those observed in the current studies (Fig. 2, right kidney) when indomethacin was adminis-

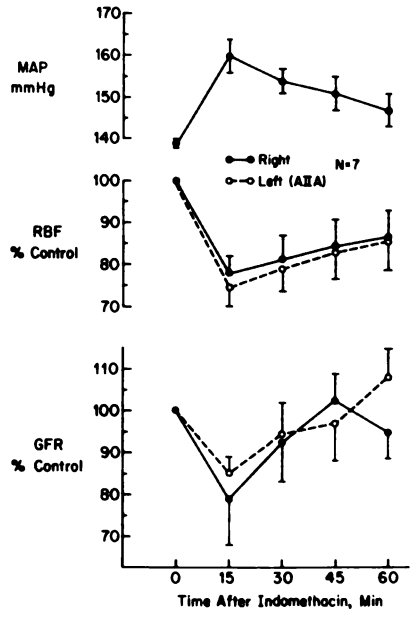

FIG. 2. Effect of left kidney angiotensin II blockade (AIIA) on the renal vasoconstrictor response to systemic indomethacin, N = 7.

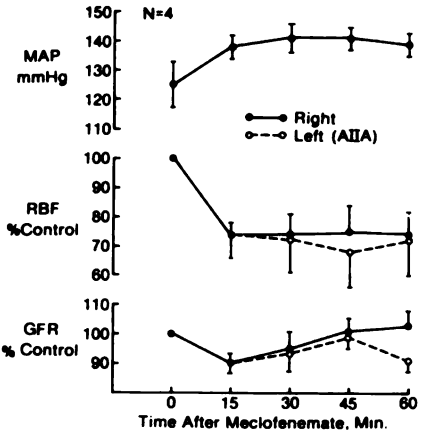

FIG. 3. Effect of left kidney angiotensin II blockade (AIIA) on renal vasoconstrictor response to systemic meclofenemate, N = 4.

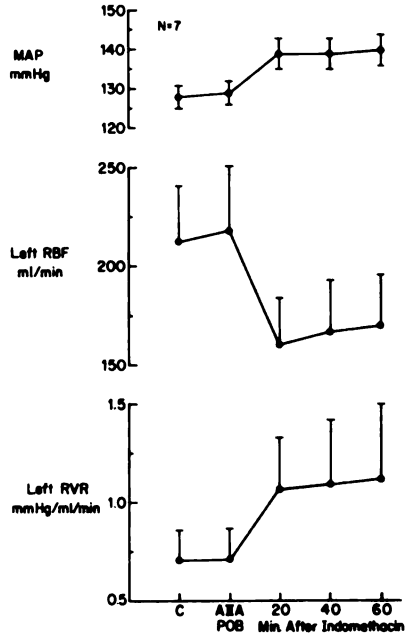

FIG. 4. Effect of combined renal α-adrenergic receptor blockade with phenoxybenzamine (POB) and renal angiotensin II blockade (AIIA) on the renal vasoconstrictor response to systemic indomethacin, N = 7.

ıe. Identical results were obtained in
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)constriction observed after systemic
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ıion. The interactions between ad-
stimuli and various vasoactive sub-
re complex. It has been well docu-
hat renal nerve stimulation or exog-
E causes the renal release of renin
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teractions between PG and adrener-
ıli in the renal vascular bed are spe-
ndent. In the isolated perfused rab-
bit kidney, exogenous PG administration decreased the renal vascular response to RNS whereas, in the isolated perfused rat kidney, the response was increased; the renal vascular response to exogenous NE was unaffected in both species (7). Using indomethacin Zimmerman and colleagues (8) examined the role of endogenous PG in the control of vascular responses in the perfused dog paw. It was found that indomethacin potentiated the cutaneous vascular responses to both nerve stimulation and exogenous NE. In the dog (11), rabbit (12), and cat (13) kidney, intrarenal infusion of PGE_2 abolished the renal vasoconstrictor response to RNS but had little effect on the renal vasoconstrictor response to exogenous NE. Additional studies in the feline kidney by Chapnick and colleagues (15) demonstrated that indomethacin enhanced the renal vascular response to exogenous NE but not RNS whereas meclofenemate had no effect on the renal vascular response to either exogenous NE or RNS. In conscious dogs, Swain and colleagues (33) observed that the increase in RVP produced by intravenous methoxamine, an α-adrenergic agonist, was increased by prior treatment with indomethacin. Our findings that renal vascular responses to exogenous NE were increased by indomethacin (Fig. 1) in anesthetized dogs are in agreement with the results of Swain and colleagues in the dog (32) and Chapnick and colleagues in the cat (15). Our findings that indomethacin did not affect renal vascular responses to RNS and meclofenemate did not affect renal vascular responses to RNS or exogenous NE are in agreement with similar observations by Chapnick and colleagues in the cat kidney (15). It is not clear why indomethacin but not meclofenemate potentiated the renal vascular responses to exogenous NE but it may be that the indomethacin effect is related to an action of the drug separate from its action to inhibit prostaglandin synthesis. These data suggest that endogenous prostaglandins do not serve to modulate the effects of the sympathetic nervous system on the canine renal vascular bed.

Mimran and colleagues (17) implicated the renin–angiotensin system as the mediator of the increase in MAP, decrease in RBF, and increase in RVR observed in the rat following

indomethacin administration. Bilaterally nephrectomized rats did not demonstrate an increase in MAP when given indomethacin. Systemic administration of AIIA attenuated the increase in MAP and prevented the decrease in RBF. Satoh and Zimmerman (33) administered indomethacin or meclofenemate into the renal artery of anesthetized dogs and did not observe an increase in MAP or renal vasoconstriction unless the renal artery was partially occluded. The renal vasoconstriction seen under these circumstances was abolished by renal blockade to angiotensin II. Our studies differ from these previous studies in that the prostaglandin synthesis inhibitors were administered systemically to anesthetized dogs. This intervention regularly produced an increase in MAP and a decrease in RBF, an observation previously made by us (28) as well as others (16, 34) in the anesthetized dog. The failure of Satoh and Zimmerman (33) to observe an increase in MAP or renal vasoconstriction in anesthetized dogs may relate to the administration of the prostaglandin synthesis inhibitors into the renal artery rather than systemically. Alternatively, they suggested that a background level (i.e., above the basal state) of circulating angiotensin II produced by the renal artery occlusion may be necessary for an increase in MAP and a decrease in RBF to occur after intrarenal administration of prostaglandin synthesis inhibitors. Our studies indicate that the renal vasoconstrictor response to systemic administration of prostaglandin synthesis inhibitors in anesthetized dogs is not mediated by the intrarenal action of the prevailing circulating levels of angiotensin II.

Since we observed that indomethacin enhanced the renal vascular response to exogenous NE, the possibility remained that the renal vasoconstriction observed after indomethacin could be mediated by the removal of endogenous PG inhibition of circulating NE. However, addition of renal α-adrenergic receptor blockade to renal AII blockade did not alter the characteristic renal vasoconstriction observed after indomethacin. Since meclofenemate did not alter the renal vascular response to exogenous NE, it was anticipated that addition of renal α-adrenergic receptor blockade to renal AII blockade would not alter the characteristic renal vasoconstriction observed after meclofenemate; such was the case. It is evident, however, that the anesthetized operated dog has higher levels of renal prostaglandin secretion than are observed in the unanesthetized conscious dog (35). Several studies have demonstrated that indomethacin or meclofenemate administration to unanesthetized conscious dogs does not result in renal vasoconstriction independent of changes in either renal venous prostaglandin concentrations (35) or in renal PGE_2 secretion or excretion (24).

In the anesthetized operated dog, not only are renal prostaglandin secretion rates elevated (35) but circulating levels of angiotensin II (36), as well as sympathetic nervous system activity, is increased. The possibility existed that the renal vasoconstrictor response to the systemic administration of indomethacin or meclofenemate to anesthetized operated dogs was due to the unopposed renal vasoconstrictor action of elevated levels of circulating angiotensin II or catecholamines. Our studies indicate that this is not the case and suggest that the renal vasoconstriction is related to the removal of endogenous prostaglandin renal vasodilator activity.

It should be emphasized that these observations pertain to the experimental circumstances of the anesthetized unstressed animal. In the anesthetized animal subjected to hemorrhage, it is clear that the resultant renal vasoconstriction, mediated by both angiotensin II and the renal sympathetic nerves, is significantly enhanced by the administration of prostaglandin synthesis inhibitors (36).

Summary. In the anesthetized dog, neither renal angiotensin II blockade nor systemic administration of meclofenemate altered the renal vascular response to renal nerve stimulation or exogenous norepinephrine. Systemic administration of indomethacin enhanced the renal vascular response to exogenous norepinephrine but not to renal nerve stimulation. Renal blockade to angiotensin II, alone or combined with renal α-adrenergic receptor blockade produced by phenoxybenzamine did not affect the renal vasoconstrictor response to systemically administered indomethacin or meclofenemate. These data demonstrate that neither endogenous circulating angiotensin II nor prostaglandins significantly alter the effects of the sympathetic

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Indomethacin Increases Renal Vascular Resistance after Adrenergic Interruption in the Dog (40628)[1]

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Prostaglandin (PG) synthetase inhibition variably affects renal hemodynamics. For example, several investigators (1–4) have demonstrated that indomethacin, a drug inhibiting the conversion of arachidonic acid to PGs, produces a marked increase in renal vascular resistance (RVR) and a decrease in renal blood flow (RBF) in anesthetized and surgically stressed dogs. In contrast, other investigators (5–7) have shown that indomethacin does not alter RVR and RBF in the nonstressed dog. Zins (6) hypothesized that the different results could be related to the level of autonomic activity existing prior to indomethacin treatment. In the stressed dog the high level of autonomic activity may lead to increased production of renal PGs which oppose adrenergic vasoconstrictor effects on renal vascular tone. Thus, PG synthetase inhibition in the stressed dog could result in increased RVR and decreased RBF. In contrast, autonomic activity and, therefore, PG levels, may be low in the nonstressed animal. Thus indomethacin infusion in these animals would have little effect on renal hemodynamics. The experiments reported here were conducted to test this hypothesis. The renal vascular response to the PG synthetase inhibitor, indomethacin, was studied in the anesthetized, surgically stressed dog before and after α-adrenergic blockade or renal denervation.

Materials and methods. Twenty-seven mongrel dogs (14 to 26 kg) were anesthetized with pentobarbital sodium, 30 mg/kg i.v. Ventilation was maintained at a constant frequency and tidal volume. Either kidney was approached through a flank incision and an electromagnetic flow transducer (Biotronex) placed around the renal artery. Electrically meaned femoral arterial blood pressure (MAP) and RBF were recorded on a Beckman Model R411 oscillograph. Zero flow was determined by occluding the renal artery distal to the flow probe. The kidney studied was removed and weighed at the end of the experiment.

Two series of experiments were performed. The effects of α-adrenergic blockade on the renal vascular response to indomethacin was studied in Series I. Nine control dogs received only indomethacin while 11 dogs received indomethacin after α-blockade. Indomethacin 0.1–0.2 mg/(kg × min) was infused over 30 min through a 20-gauge needle positioned directly in the renal artery distal to the flow probe. The total dose was dissolved in 100 ml of 22 m*M* sodium bicarbonate–isotonic saline (pH 8.3). This dosage of indomethacin exceeds that reported to inhibit prostaglandin synthesis (2). Arachidonic acid, 10 mg in 1 ml ethanol, was given i.v. as a bolus before and 30 min following indomethacin. Attenuation of the expected decrease in MAP due to arachidonic acid was interpreted as an indication of inhibition of PG synthesis. α-Adrenergic blockade was induced by phenoxybenzamine hydrochloride (Dibenzyline) at a dose of 1.6–2.3 μg/(kg × min), $n = 5$, or a dose of 50 μg/(kg × min), $n = 6$, both doses given into the renal artery over 20 min. The total dose was diluted with 20 ml normal saline. Phenoxybenzamine infusion was completed at least 20 min prior to infusion of indomethacin. Efficacy of α-blockade was tested by injecting a 1 or 4 μg bolus of L-norepinephrine bitartrate (Levophed) into the renal artery before and after phenoxybenzamine. Norepinephrine, as the base, was diluted in 1 ml normal saline. The criterion for α-blockade was a marked attenuation of the expected decrease in RBF in response to norepinephrine. To test the response to the indomethacin vehicle, 13 Series I dogs received a bicarbonate–saline infusion prior to the indomethacin infusion.

The effect of renal denervation on the renal vascular response to indomethacin was stud-

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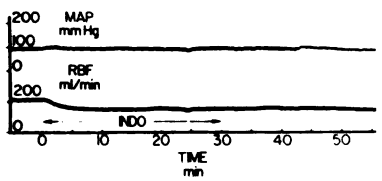
Series II. In seven dogs the response to ıethacin in an innervated kidney was ıred to the response in the same kidney ays after denervation. Observations in nervated kidney were made identically se in Series I except that aseptic condi- were maintained and indomethacin was d into a peripheral vein to eliminate the ility of postoperative hemorrhage from nal artery. The exposed kidney was lecapsulated and denervated by strip- the renal artery, vein, and proximal . Decapsulation facilitated the dener- . The artery and vein were painted with enol. Penicillin G combined with dih- reptomyocin (Combiotic) was admin- l i.m. for 4 days following surgery.

r data was collected from the dener- kidney, both the denervated kidney e contralateral innervated kidney were y removed and weighed. Slices were in liquid nitrogen and stored at −180° ssayed for norepinephrine content by pectrophotofluorometric method of ne (8).

ll dogs RBF reached a low value by the int of the indomethacin infusion (ap- 17 min) and remained at this level hout and for at least 15 min following usion (Fig. 1). Average MAP and mean were obtained by dividing this steady- time interval into the planimetrically ined area under the pressure–time or the flow–time curve over this inter- verage MAP and RBF were used to ate RVR. Statistical comparison of re- s to pharmacologic interventions were within groups and between groups us- udent's paired and nonpaired t tests, tively. Because preindomethacin values d significantly between the α-blocked and control groups, experimental data were expressed in percentage of preindomethacin values. P values <0.05 are considered indicative of statistical significance. Data are expressed as mean ± SEM.



1. Representative experimental record of the urse of mean arterial blood pressure (MAP) and lood flow (RBF) changes associated with indo- in (INDO) infusion. Arrows indicate the begin- d end of infusion.

Results. In Series I, one of two different doses of phenoxybenzamine was used to produce blockade. Renal arterial administration of 1 or 4 μg of norepinephrine reduced RBF 31 or 78%, respectively ($P < 0.01$), prior to phenoxybenzamine, but had no significant effect on flow ($P > 0.10$) following α-blockade with phenoxybenzamine. Since there were no statistical differences in the responses to indomethacin for dogs receiving 1.6–2.3 μg/(kg × min) and those dogs receiving 50 μg/(kg × min) of phenoxybenzamine, these data were combined to make up the α-blocked group.

Figure 2A shows the effect of indomethacin infusion on RVR, RBF, and MAP in the α-blocked and the control groups of Series I. Prior to indomethacin, RVR in the α-blocked dogs was 0.28 ± 0.04 as compared to 0.44 ± 0.06 (mm Hg·min·100 g)/ml in the control dogs ($P < 0.05$). In response to indomethacin, RVR increased an average of 22% in both the α-blocked group ($P < 0.001$) and the control group ($P < 0.005$) while RBF decreased an average of 18% in the α-blocked ($P < 0.005$) and 16% in the control ($P < 0.005$). The percentage changes in RVR and RBF did not differ between the two groups of dogs ($P > 0.50$). MAP was unaffected by indomethacin in either group ($P > 0.50$). Indomethacin-free sodium bicarbonate–saline solution had no effect on measured variables.

Responses to indomethacin infusion before and 5–6 days following renal denervation, Series II, are shown in Fig. 2B. Although preindomethacin RVR in the intact Series II kidney appears higher than RVR in the Series I control kidney both before and after indomethacin, the differences were not significant. Indomethacin increased RVR an average of 23% before ($P < 0.05$) and 35% after ($P < 0.005$) renal denervation and decreased RBF an average of 19% before ($P < 0.01$) and 25% following denervation ($P < 0.025$). The changes in RVR and RBF associated with indomethacin did not differ between the innervated and denervated kidneys ($P > 0.20$). MAP was not changed ($P > 0.10$) by indo-

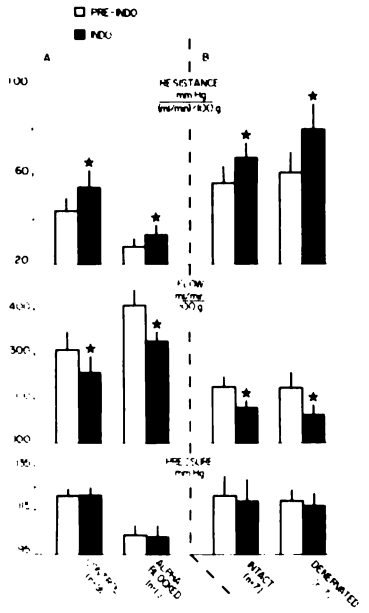


FIG. 2. Renal vascular resistance, renal blood flow, and mean arterial pressure responses to indomethacin 0.1–0.2 mg/(kg/min) × 30 min. (A.) Series I: Responses to renal arterial infusion of indomethacin in α-blocked and in control dogs. (B.) Series II: Responses to intravenous infusion of indomethacin in the same kidneys before and following renal denervation, n = number of dogs. The star indicates significant difference from preindomethacin value ($P < 0.05$).

methacin.

In seven experiments, denervation reduced renal norepinephrine content ($P < 0.001$) from 0.703 ± 0.052 in the innervated kidney to 0.136 ± 0.008 μg/g wet wt in the contralateral denervated kidney. Recovery of norepinephrine, estimated by use of an internal standard was 67.1% ± 2.01. In nine trials in six dogs (three trials prior to and six following renal denervation), arachidonic acid decreased MAP 24% ($P < 0.001$) prior to indomethacin but did not change MAP ($P > 0.10$) following indomethacin infusion.

Discussion. Our studies do not support the hypothesis that indomethacin increases RVR by eliminating PG modulation of sympathetic vasoconstrictor activity in the anesthetized, surgically stressed dog. α-Adrenergic blockade, with doses of phenoxybenzamine sufficient to eliminate the decrease in RBF in response to norepinephrine, did not diminish the increase in RVR in response to indomethacin. Furthermore, surgical renal denervation failed to attenuate the renal vasoconstrictor response to indomethacin although it reduced the norepinephrine content of the kidney to ⅛ that of the innervated control. Indomethacin eliminated the decrease in MAP seen in response to arachidonic acid, a PG precursor, thus providing evidence for inhibition of PG synthesis. The validity of the hypothesis that renal adrenergic activity is modulated by PGs would seem to depend upon verification of PG secretion as a function of renal sympathetic activity. Although PG release in response to renal sympathetic stimulation has been reported in several species (9, 10), McGiff *et al.* (11) reported no increase in canine renal vein PGE with renal nerve stimulation.

Our observation that the effect of indomethacin on RVR was not altered by adrenergic interruption is in agreement with the finding of Chapnick *et al.* (12) that α-adrenergic blockade does not attenuate the rise in RVR in response to indomethacin in the feline kidney. Their studies, like ours, were performed in presumably normovolemic animals. The data of Henrich *et al.* (13) indicates that indomethacin is equally effective in reducing RBF in both denervated and innervated kidneys of anesthetized, surgically stressed dogs under normovolemic conditions. However, in the same study renal denervation attenuated the reduction in RBF associated with hypotensive hemorrhage in the indomethacin-treated dog. This observation suggests an interaction between renal nerve and PG effects on renal hemodynamic responses to hypotensive hemorrhage.

The increase in RVR associated with indomethacin might be related to an interaction between PG synthesis and renal vasoconstriction caused by angiotensin II. There is evidence that angiotensin II releases PGs which diminish the renal vasoconstrictor effect of this pressor hormone (14). Terragno *et al.* (7) reported that plasma renin activity is highly correlated with renal PGE-like substances in renal venous blood of conscious or surgically stressed dogs. Furthermore, interruption of the renin–angiotensin system diminishes the increase in RVR associated with inhibition of prostaglandin synthesis in the normovolemic animal (12, 15). Recently, Henrich (16) re-

ıat antagonism of the renin–angio-
stem coupled with renal denervation
against the renal vasoconstriction as-
with hypotensive hemorrhage fol-
G synthetase inhibition.

udy shows that the effect of indo-
ı on RVR in the normovolemic dog
ndent of the adrenergic nervous sys-
: find no evidence for endogenous
nportant modulators of the adrener-
ous system in the control of RBF in
ıovolemic dog. However, our find-
ng with those cited, do not rule out
bility that indomethacin has a direct
RVR or that indomethacin increases
eliminating PG modulation of the
giotensin system.

ary. The effect of adrenergic inter-
on the renal vascular resistance
ncrease associated with the prosta-
PG) synthetase inhibitor, indometh-
s studied in anesthetized, surgically
dogs. α-Adrenergic blockade was
l with phenoxybenzamine. In re-
› indomethacin, neither the increase
nor the decrease in renal blood flow
iffered between the α-blocked (n =
he control dogs (n = 9). In a separate
experiments, the effect of renal de-
ı on the renal vascular response to
hacin was examined in the same kid-
›re and 5–6 days after that kidney
ically denervated (n = 7). The indo-
ı-associated increase in RVR and de-
RBF did not differ in the intact and
ed kidneys. Under conditions of an-
and surgical stress in normovolemic
ther α-adrenergic blockade nor renal
ion changed the renal hemodynamic
to indomethacin. We, therefore,
that under these conditions the in-
cin-induced increase in RVR is not
mediated by elimination of PG modulation of renal sympathetic vasoconstrictor activity.

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Studies on the Effect of Cyproheptadine on Growth Hormone Secretion (40629)

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There is evidence that several monoamines, including dopamine, norepinephrine, and serotonin, have roles in regulating pituitary growth hormone (GH) secretion (1–3). Evidence on the role of serotonin remains unclear. Nakai *et al.* (4) and Smythe *et al.* (5) reported that 5-hydroxytryptophan administered to humans stimulated GH secretion, but Handwerger *et al.* (6) failed to confirm this observation. Much of the evidence that serotonin directly influences GH release has been derived from indirect experiments in which so-called specific serotonin-receptor antagonists have been used. In this study we investigated the effect of serotonin and cyproheptadine on GH synthesis and release by normal rat pituitary glands and by dispersed pituitary tumor cells.

Materials and methods. Female and male rats (Wistar–Furth, 200–220 g) were housed four to five per cage in a temperature-controlled room (22–23°) under artificial illumination (0600–1800 hr). Tumor homogenate from mixed prolactin (PRL-)- and GH-secreting tumor, MtTW15 was injected into the scapular region 6–8 weeks before the animals were studied, as described previously (7).

For *in vivo* experiments on hemipituitary glands, the animals were decapitated at 1000 hr and the anterior pituitary was quickly removed and bisected. Flasks containing three hemipituitary glands from different rats were incubated in 1 ml of tissue culture medium 199 (Microbiological Associates, Inc.) containing 10 μCi [4,5-^{3}H]leucine (30 Ci/mmole). Each group contained four flasks. The flasks were incubated at 37° in a Dubnoff shaker in an atmosphere of 95% O_2, 5% CO_2 (vol/vol). After 5-hr incubation the medium was removed and the glands were rinsed and homogenized in 1 ml distilled water containing 1% Triton X-100. Aliquots of the incubation medium and of pituitary homogenates were subjected to polyacrylamide gel electrophoresis (8). The separating gel was 7% acrylamide, pH 8.9, and the electrode compartments contained 0.5 *M* Tris–glycine buffer, pH 8.3. Duplicate gels were run at 4 mA/gel for each sample. The protein bands were stained in 7% acetic acid containing 0.5% amido black 10B; excess stain was removed electrophoretically. The location of GH and PRL on the gels was ascertained by subjecting NIH reference hormones to the same electrophoretic separation. The stained protein bands were cut from the gel columns and placed in scintillation vials containing 1 ml of NCS (tissue solubilizing agent; Amersham Searle) and water solution (92:8, vol/vol). A toluene-based scintillation fluid was added to each vial at room temperature and the samples were counted in a liquid scintillation spectrometer. The total amount of newly synthesized GH was computed as the amount released into the medium plus that found in the pituitary gland.

Isolated pituitary tumor cells were prepared by mincing 5 g of the MtTW15 tumor in 10 ml medium 199 containing 1.5 mg/ml trypsin (Worthington) and 100 μg/ml bovine serum albumin. Tissue fragments were incubated for 20 min at 37° under an atmosphere of 95% O_2, 5% CO_2. Following incubation the tissue fragments were brought to room temperature and then drawn up and expelled 25 times through a plastic Pasteur pipet. The debris and large fragments were allowed to settle 5 min and the supernatant containing the isolated cells was aspirated. Trypsin inhibitor (Worthington) was added to this suspension (1.5 mg/ml). The acutely dispersed cells were pipetted into plastic vials (final volume 1 ml) and incubated for either 2.5 or 5 hr in a Dubnoff shaker at 37° in an atmosphere of 95% O_2, 5% CO_2, followed by centrifugation at 3500*g* for 10 min. After centrifugation the medium and cell pellet were separated and stored.

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e tumor cell content of the incubation
ım was counted in a hematological
ing chamber. The percentage of viable
as determined by trypan blue exclusion,
)5% prior to and 90% after the 5-hr
ation.

e incubation medium and rat serum GH
nt was measured by a double-antibody
immunoassay using materials and pro-
supplied by the NIAMDD Rat Pitui-
lormone Distribution Program. The ref-
e hormone used was GH-RP-1 growth
one.

e results are expressed as mean ± SEM
statistical analysis was performed by
sis of variance.

ults. The intraperitoneal administration
proheptadine (3 mg/kg) decreased se-
GH in normal female rats: 15 min after
ion serum GH was significantly ($P <$
suppressed compared with control val-
able I). The pituitary glands from these
ls were removed 15, 30, and 45 min
cyproheptadine injection. *In vitro* re-
of both radioimmunoassayable GH and
synthesized [^{3}H]GH was significantly
ited from pituitary glands taken from
ls 45 min after injection (Table II).

E I. THE EFFECT OF CYPROHEPTADINE ON THE SECRETION OF GROWTH HORMONE *IN VIVO*[a]

Minutes after injection (min)	Serum growth hormone (ng/ml)
0	42.6 ± 14.5
15	11.7 ± 1.7*
30	23.6 ± 5.3
45	22.1 ± 3.5

e 3 mg/kg cyproheptadine was injected intraperly in female rats, six rats per group; mean ± SEM.
< 0.01 versus control.

Direct effects of cyproheptadine on pituitary GH synthesis and release were investigated. Cyproheptadine (60–600 n*M*) did not affect GH synthesis (Table III, Expts II and III) or release (Table III, Expts II and III). However, the same concentrations of cyproheptadine produced a dose-dependent inhibition of PRL release *in vitro* (Fig. 1). Serotonin (0.1–10 μM) and dopamine (500 n*M*) were without effect (Table III) on GH release.

The effect of cyproheptadine on GH secretion by a transplantable GH-secreting pituitary tumor was also investigated. It was previously shown that the transplantable pituitary tumor MtTW15 secretes large quantities of both GH and PRL (9). During 5-hr incubation of trypsin-dispersed pituitary tumor cells, a linear release of growth hormone and prolactin was observed (Fig. 1). The addition of dibutyryl-cAMP (5 m*M*) significantly increased GH release ($P < 0.05$), while cyproheptadine (6–12 μM) inhibited the release of the hormone ($P < 0.01$). Interestingly, the secretion of GH and PRL by these isolated tumor cells in response to dbcAMP and cyproheptadine showed a parallel pattern (Fig. 2).

The effect of chronic administration of cyproheptadine (3 mg/kg subcutaneously for 5 days) on serum GH and PRL, tumor growth, and body weight was investigated in rats bearing the MtTW15 tumor. Cyproheptadine injections did not affect the spontaneous increase in serum GH and serum PRL as they occurred in tumor-bearing controls (Table IV). Similarly cyproheptadine administration did not have an effect on body weight and tumor size. At the end of the 5-day period the animals were sacrificed in order to compare the effect of the administration of cyprohep-

E II. THE EFFECT OF CYPROHEPTADINE INJECTION ON THE *IN VITRO* SECRETION OF GROWTH HORMONE[a]

	Incorporation of [^{3}H]leucine into growth hormone (cpm/mg pituitary gland)		Growth hormone measured by radioimmunoassay (μg/mg pituitary)
	Pituitary gland	Medium	Medium
l	11750 ± 590	970 ± 80	6.60 ± 0.42
after cyproheptadine injection	11690 ± 350	850 ± 30	6.68 ± 0.25
after cyproheptadine injection	11210 ± 630	840 ± 70	6.36 ± 0.36
after cyproheptadine injection	11160 ± 530	540 ± 60*	5.36 ± 0.36*

e 3 mg/kg cyproheptadine was injected in female rats and three hemipituitary glands were incubated 15, 30, min after injection. Four flasks per group; mean ± SEM.
< 0.05 vs. control.

TABLE III. Effects of Serotonin, Cyproheptadine, and Dopamine on Growth Hormone Secretion by Normal Pituitary Glands *in Vitro*[a]

	Incorporation of [^{3}H]leucine into growth hormone (cpm/mg pituitary gland)		Growth hormone measured by radioimmunoassay (μg/mg pituitary)
	Pituitary gland	Medium	Medium
Expt. I (male rats)			
Control	8055 ± 960	2830 ± 550	15.16 ± 1.13
Serotonin 0.1 μ*M*	8080 ± 1210	2770 ± 540	15.44 ± 1.57
1 μ*M*	9210 ± 710	2790 ± 600	15.58 ± 0.89
10 μ*M*	9250 ± 210	2650 ± 200	13.68 ± 2.00
Expt II (female rats)			
Control	9325 ± 760	365 ± 70	6.13 ± 0.47
Cyproheptadine 60 n*M*	8930 ± 1230	370 ± 60	5.65 ± 0.61
240 n*M*	9920 ± 510	395 ± 70	6.04 ± 0.52
600 n*M*	9590 ± 600	360 ± 25	5.71 ± 0.85
Expt III (male rats)			
Control	8960 ± 645	2430 ± 200	13.60 ± 1.26
Cyproheptadine 100 n*M*	9045 ± 766	2455 ± 105	14.05 ± 1.05
Expt IV (female rats)			
Control	4640 ± 470	370 ± 100	2.62 ± 0.21
Dopamine 500 n*M*	4740 ± 320	395 ± 100	2.64 ± 0.31

[a] Four flasks per group; mean ± SEM.

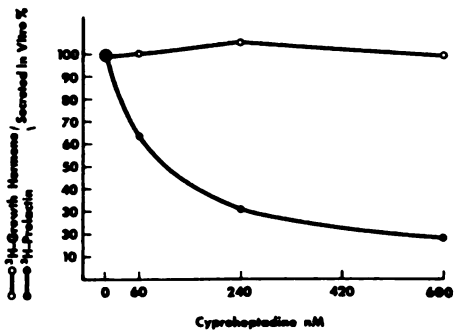


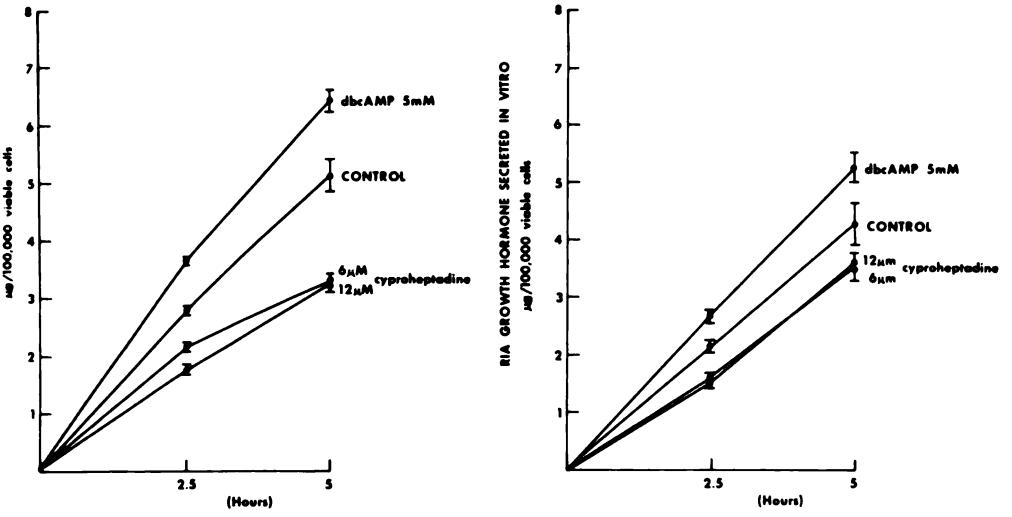
FIG. 1. Effect of cyproheptadine *in vitro* on the secretion of radioactive prolactin and growth hormone. Hemipituitary glands from three different rats were incubated in medium 199 containing [^{3}H]leucine for 5 hr as described. Aliquots of the incubation medium were subjected to polyacrylamide gel electrophoresis and the radioactivity in the stained bands of prolactin and growth hormone measured. Four flasks per group.

tadine on organ weights. Chronic cyproheptadine administration did not affect the organomegaly (liver, spleen, adrenal) which occurs as a reaction to the hypersecretion of hormones by the mixed GH-PRL producing tumor MtTW15.

Discussion. The evidence for a positive role of serotonin on growth hormone secretion is partly derived from indirect experiments in which so-called specific serotonin antagonists such as cyproheptadine are used. In man, cyproheptadine suppresses sleep-related GH release (10) and inhibits in normal individuals the release of GH induced by insulin hypoglycemia (11, 12), arginine infusion (4), physical exercise (13), and glucagon (14). In normal children the mean GH response to an oral glucose administration was significantly reduced by cyproheptadine (14). Systemic administration of cyproheptadine suppressed GH secretion in neonatal rats (15). Our findings of suppressed GH secretion within 15 min after the administration of cyproheptadine agree with these observations. Following systemic cyproheptadine administration pituitary GH release was significantly inhibited.

This action of cyproheptadine was shown not to be exerted directly at the level of the pituitary gland. In addition, serotonin by itself had no effect on pituitary GH synthesis and release *in vitro*. The neuropharmacological specificity of cyproheptadine is uncertain. In addition to antiserotoninergic actions of the drug, histamine-, choline-, and dopamine-blocking actions have been described. The ability of cyproheptadine to block L-dopa-induced GH release might indicate an antidopaminergic action of the drug on GH secretion (3, 16).

The evidence for a positive role of dopamine as a hypothalamic regulator of GH



IG. 2. Effect of DBcAmp and cyproheptadine on prolactin and growth hormone secretion by trypsin-isolated tary tumor cells *in vitro*. Approximately 0.5 × 10^6 cells were incubated for the indicated times in the presence of AMP or cypropeptadine.

TABLE IV. THE EFFECTS OF CYPROHEPTADINE ON SERUM GROWTH HORMONE AND PROLACTIN IN RATS BEARING A MIXED GROWTH HORMONE/PROLACTIN-SECRETING TUMOR (MtTW15)[a]

	Serum growth hormone (μg/ml)		Serum prolactin (μg/ml)	
	Day 1	Day 5	Day 1	Day 5
rol tumor-bearing rats	2.28 ± 0.30[b]	3.13 ± 0.74	11.71 ± 1.71	13.90 ± 2.69
oheptadine-injected tumor rats	2.93 ± 0.14	3.87 ± 0.64	13.80 ± 1.39	18.00 ± 2.64

Six MtTW15-bearing rats per group; cyproheptadine (3 mg/kg; s.c.) was administered for 5 days; 90 min after ast injection the animals were killed by decapitation and blood was collected.
Mean ± SEM.

etion is strong. Low concentrations of the amine receptor agonists apomorphine l piribedil have been reported to stimulate l secretion both in man (17, 18) and in rats). The ability of the specific dopamine eptor antagonist haloperidol to reduce sma GH levels and to block apomorphine-uced GH release in rats (19) strengthens hypothesis. Recently comparable human a have been reported in which pimozide s able to block GH release induced by rcise and arginine infusion (13).

Dopamine probably exerts its effect by in-iting somatostatin release in the external er of the median eminence (20). Further dence for the role of dopamine is found in observations of a positive correlation be-een the diurnal variations in plasma GH d hypothalamic dopamine content (21). ar experiments also support the view that dopamine acts as a neurotransmitter at the hypothalamic level to regulate GH secretion. We have shown previously that neither norepinephrine, epinephrine, ergocryptine, nor perphenazine has a direct effect on GH synthesis and release by the normal pituitary gland *in vitro* (22, 8). The 500 n*M* dopamine, a concentration which inhibits PRL secretion by the pituitary gland by 85% (23), was shown to have no effect on GH release *in vitro*. This contrasts with observations by Quijada *et al.* (24) who showed a significant inhibitory effect of dopamine (up to 10^{-5} *M*) on *in vitro* pituitary GH secretion.

The direct inhibitory effect of cyproheptadine on GH secretion by dispersed rat pituitary tumor cells has not been described before. Cyproheptadine administration (0.6 mg daily for 2 days) decreased plasma GH concentrations during oral glucose tolerance tests

in four of six patients with active acromegaly (25), but Chiodini *et al.* (26) did not observe a change in plasma GH in response to the administration of a single dose of 8 mg cyproheptadine, nor during chronic treatment of four acromegalic patients for 2 weeks with 12 mg cyproheptadine per day. We have shown before that cyproheptadine has a direct inhibiting effect on prolactin secretion by the normal pituitary gland *in vitro* (27): This effect was shown not to be mediated via a serotonin- or dopamine-receptor mechanism. The observation that cyproheptadine had no direct effect on GH secretion by the pituitary gland *in vitro*, while it does inhibit secretion of GH and PRL by dispersed pituitary tumor cells *in vitro*, suggests that the GH secretion in this mixed GH-PRL-secreting pituitary tumor has acquired the properties of PRL-secreting tumor cells. However, chronic administration of cyproheptadine did not affect tumor growth or serum GH in rats bearing the transplantable pituitary tumor MtTW15.

Summary. The administration of cyproheptadine to female rats transiently decreased serum GH levels. Forty-five minutes after cyproheptadine injection, *in vitro* GH synthesis and release was significantly decreased. Although cyproheptadine decreased PRL release *in vitro*, the drug had no direct *in vitro* inhibitory effect on GH release. Cyproheptadine did not affect the secretion of GH and PRL from transplantable tumor MtTW15, although it inhibited the *in vitro* release of GH from dispersed tumor cells. Cyproheptadine had no effect on either tumor size or the biological actions produced by the tumor hormones.

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Hydrolytic Enzymes of the Ischemic Kidney (40630)

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al ligation of the aorta between the teries in the rat induces rapidly pro- malignant hypertension as well as trophy of the ischemic kidney (1–4). hology of this kidney is characterized lar changes in virtually all renal struc- here is widespread metaplasia of ar- smooth muscle cells into juxtaglo- cells (3), simple atrophy of the outer tubular cells, and atrophy, hyperpla- polyploidy in the inner cortex (pars 5). In the cortex, these morphological ons are accompanied by an increase and RNA with a decrease in proteins

present study indicates that not only 5–8) but also certain hydrolytic en- ctivities, such as those of acid phos- , β-glucuronidase, and cathepsin D, eased in the ischemic renal cortex. As by their distribution pattern following ic centrifugation, these enzymes ap- t to be localized, like renin, in juxta- ular cell granules but rather in cortical cell lysosomes.

rials and methods. Animals and sur- male Sprague–Dawley rats (Fermes oratoires Canadiens d'Elevage Ltée, tant, Québec, Canada) were used in es of experiments. Surgery always was ied under ether anesthesia on groups nimals, on the 1st day of the experi- with the animals being sacrificed on 1 day.

first series was done on rats with an verage body weight of 200 g (range: 210 g). Surgery consisted of partial of the aorta between the renal arter- g a silk thread and the style (diameter: m) of a No. 30 hypodermic injection as described elsewhere (3). These an- together with control, unoperated were taken for histopathology, elec- croscopy, and biochemical analysis of the left ischemic kidney: determination of renin, acid phosphatase, β-glucuronidase, cathepsin D, and protein content; isopycnic centrifugation of renal cortical homogenates; and electron microscopy of fractions obtained by centrifugation.

The second series of experiments was performed on rats with an average initial body weight of 215 g (range: 205 to 225 g) to compare the effects of styles of larger diameter on renal atrophy, induction of hypertension, and β-glucuronidase activity of the renal cortex. Since preliminary investigations had shown that a style larger than 0.740 mm does not induce any noticeable atrophy, the final study included styles ranging from 0.103 to 0.740 mm in diameter as well as an unoperated control group. All groups were made up of at least 20 animals. The heart and left kidney of 10 animals of each group were fixed in Bouin–Hollande fluid for 24 hr and then weighed. These kidneys were utilized for histopathological study. The left renal cortex of an additional 10 rats in each group was used for determination of β-glucuronidase activity.

Histopathology. After embedding in paraffin, the left kidney was cut longitudinally at 5 μm and stained according to the PAS technique.

Homogenization and ultracentrifugation. The left renal cortex was homogenized in an 0.25 *M* ice-cold sucrose solution, employing a Potter–Elvehjem apparatus at 800 rpm with three up-and-down strokes. The suspension was centrifuged at 1000*g* for 10 min. The sediment was resuspended in 0.25 *M* sucrose, homogenized with two strokes, and centrifuged for 10 min at 500*g*. The two supernatants were pooled and made up to volume with 0.25 *M* sucrose to form a 1:5 homogenate. Each homogenate was tested separately for protein and hydrolytic enzyme activity, while pooled homogenates were first tested

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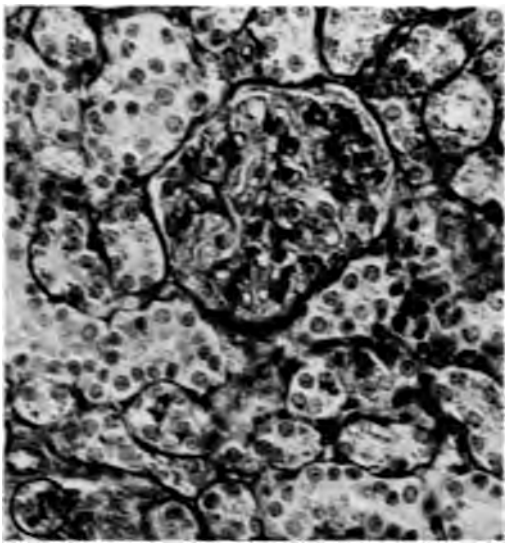

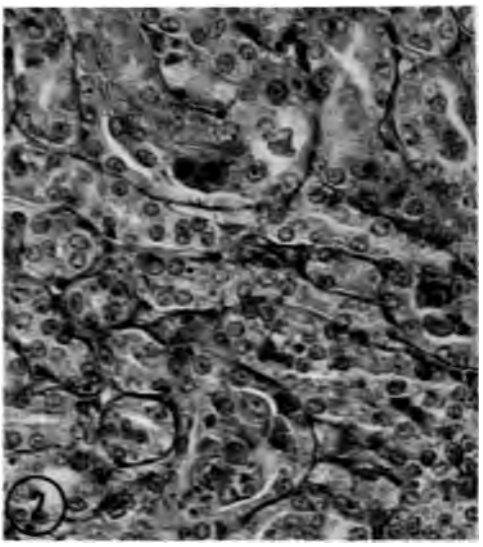

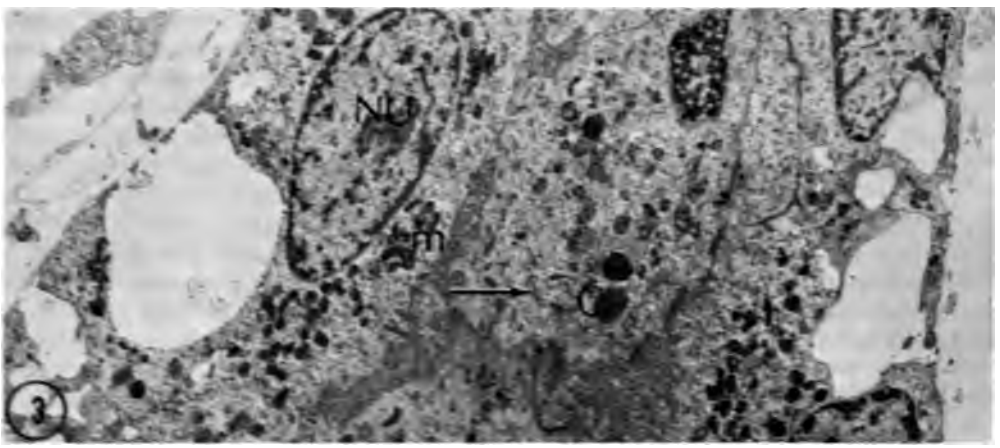

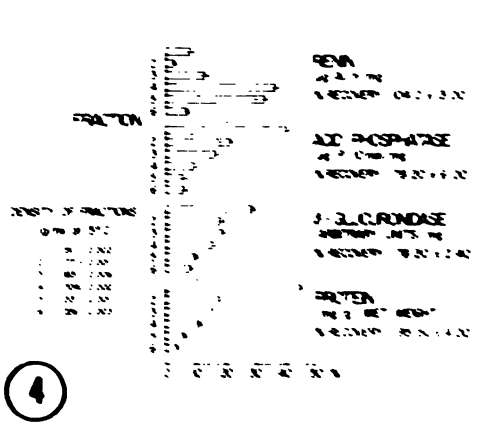

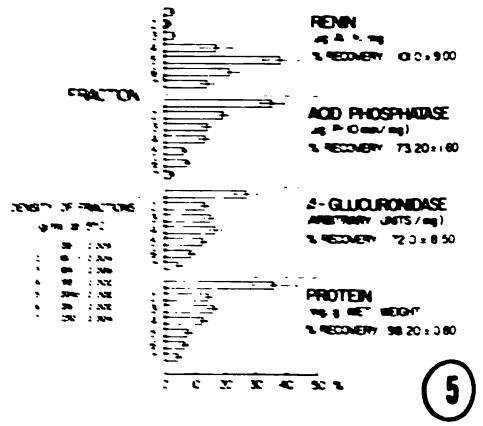

FIGS. 1–5 (1) Severely ischemic outer cortex (style diameter 0.103 mm). All the proximal convoluted tubules have lost their lumina and brush border. Note the regularity of size, shape, and staining intensity of tubular cell nuclei (PAS × 332.5).

tein and enzymes (hydrolytic enzymes in) and then fractionated by isopycnic igation in a sucrose density gradient ng to the method of Morimoto *et al.* llowing centrifugation, the bands were d by aspiration and assayed for pro- zyme activity, and density (at 5°).

me assays. β-Glucuronidase and acid atase activities were assessed by the lology of Gianetto and DeDuve (10), hepsin D activity by the Barrett tech- 11). Renin activity was measured with lioimmunoassay method of Haber *et* for the generation of angiotensin I, as d by Gross and Barajas (13) for renal homogenates and fractions. A com- Angiotensin I Immutope Diagnostic t Kit (E. R. Squibb and Sons, New was used. Plasma (substrate) for the a was obtained from 24-hr bilaterally ctomized, 200-g female, Sprague– rats. Incubation of homogenates and s with substrate was done at pH 7.4 r. The reactions were linear.

in determinations. The protein content renal cortex was measured by the ue of Lowry *et al.* (14). In all cases, dent's *t* test was used for statistical s.

ron microscopy. The technique em- for electron microscopy of the is- renal cortex and fractions has been ed earlier (3).

lts. Renal ischemia induced with a style 3 mm in diameter. Histopathology and microscopy. Outer cortex. After 10 f ischemia, atrophy was extremely l, particularly of the proximal convo- bular cells and, to a much lesser de- those of the distal tubules and cortical ng ducts (Fig. 1). In the proximal con- tubules, the lumina had collapsed brush border was often absent; the basement membranes were thickened, the basilar interdigitating processed had disappeared, and the intercellular spaces were enlarged. All the tubular cell nuclei were similar in size, contour, and staining intensity. The cytoplasm contained only a few mitochondria, prominent lipid vacuoles, and a few cytosomes.

Inner cortex. Many proximal tubular cells of the pars recta were filled with cell cords containing nuclei of irregular size, shape, and staining intensity (Fig. 2). The most striking characteristic of these cells was the hypertrophy of the protein-synthetizing apparatus: prominent nucleoli and numerous cisternae of rough endoplasmic reticulum, and free and polyribosomes. Apart from these changes, the cells were similar to those of the outer cortex. The number of cytosomes in particular was not increased (Fig. 3).

Arteries and arterioles. A great number of smooth muscle cells of arterioles, and to a lesser degree of some arteries, were replaced by cells having all the characteristics of hyperactive juxtaglomerular cells (3). There were no degenerative changes in arteries or arterioles.

Enzyme activity and protein content of the renal cortex. There was an increase in the activity of all three hydrolytic enzymes in the ischemic renal cortex, as compared with the left renal cortex from control rats, when expressed per milligram of protein (Table I). Acid phosphatase was, however, increased the least of all and was not significantly enhanced over control values, when expressed per gram of wet weight (not shown). Renin activity was elevated fivefold over corresponding values of the control renal cortex. The protein content of the ischemic renal cortex was much lower than that of the control left cortex.

Isopycnic centrifugation of renal cortical ho-

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verely ischemic inner cortex (style diameter 0.103 mm). The tubules are filled with cell cords showing ty of size, shape, and staining intensity of nuclei (PAS × 332.5).

oximal convoluted tubular cells of severely ischemic inner cortex (style diameter 0.103 mm), showing of lumen, part of brush border, a nucleus with a prominent nucleolus (NU), and a markedly simplified containing mitochondria (m), a few cytosomes (C), cisternae of rough endoplasmic reticulum (arrow), and ribosomes. Note the enlargement of the intercellular spaces (× 7847).

pycnic centrifugation of control renal cortex. Each value represents the mean ± SE of five experiments ls (five renal cortices) of homogenates.

pycnic centrifugation of ischemic renal cortex (style diameter: 0.103 mm). Each value represents the mean ive experiments using pools (8 to 10 renal cortices) of homogenates.

TABLE I. RENIN ACTIVITY, HYDROLYTIC ENZYME ACTIVITY, AND PROTEIN CONTENT OF THE CORTEX FROM THE ISCHEMIC (ENDOCRINE) KIDNEY

	Protein (mg/g wet wt)	Renin activity[a] (μg AI/hr/mg protein)	β-Glucuronidase activity (AU[b]/mg protein/10 min)	Cathepsin D activity (BU[c]/mg protein/10 min)	Acid phosphatase activity (μg P/mg protein/10 min)
Control kidney	97.8 ± 2.7	7.5 ± 0.65	15.6 ± 0.7	0.382 ± 0.002	14.5 ± 0.9
Ischemic kidney	77.1 ± 2.5*	36.4 ± 7.02*	62.8* ± 2.4	0.886* ± 0.031	19.5** ± 0.8

[a] Renin activity was measured in homogenates (pool of 5 control renal cortices and 8 to 10 ischemic renal cortices). Each value represents the mean ± SE of five experiments. Hydrolytic enzyme activity was measured in homogenates of kidneys of single rats.

[b] Arbitrary units.

[c] Barrett units.

* $P < 0.005$.

** $P < 0.01$.

mogenates. Isopycnic centrifugation of control cortical homogenates yielded six bands (Fig. 4), and the ischemic kidney seven bands (Fig. 5). The greatest activity of acid phosphatase and β-glucuronidase and the greatest amount of protein were localized in band 1 of both normal and ischemic renal cortices. There was a decrease in activity of the hydrolytic enzymes and in protein content as the density increased. Renin activity was mostly localized in bands 4 and 5 of control and in bands 4, 5, and 6 of ischemic cortices.

Electron microscopy of fractions. Control renal cortex. Fraction 1 contained small vesicles, many of which exhibited attached ribosomes; it also showed other vesicles of larger size with a flocculent, slightly granular content corresponding to lysosomes. Fraction 2 displayed the latter type of vesicles with much less rough and smooth endoplasmic reticulum. Fraction 3 was made up mostly of mitochondria. Fraction 4 revealed dense granules and other granules with a finely dispersed content of lower electron density. Fraction 5 was made up of the same type of granules but in lesser number, many empty vesicles and vacuoles being present. Fraction 6 contained a few dense or pale granules and many empty vesicles and vacuoles.

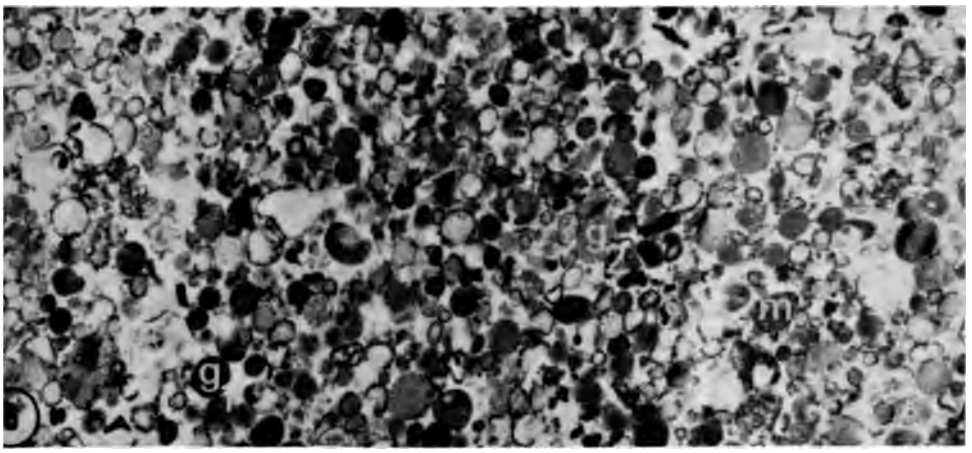
Ischemic renal cortex. Fractions 1, 2, and 3 were identical to those of the control renal cortex. Fractions 4, 5, and 6 contained mainly granules of either high or low electron density. The granules were most abundant in fraction 5 (Fig. 6) and less so in fractions 4 and 6, the rest of the fractions being made up of empty vesicles and vacuoles. Fraction 7 still contained a few dense or less dense granules and many empty vacuoles and vesicles.

Renal ischemia induced with styles larger than 0.103 mm in diameter. As can be seen in Table II, partial ligation of the aorta produced renal atrophy which was roughly inversely proportional to the diameter of the style used. The decrease in protein content of the kidney was significant in all cases, compared with control values. Heart weight was increased when expressed as mg/100 g body wt in all groups with ligated aortas. The decrease in body weight was marked in all ligated animals.

Histopathological examination of the ischemic kidney showed an identical picture, irrespective of the diameter of the style: simple atrophy of the outer cortex and hyperplasia with polyploidy in the inner cortex. These changes were evident in all ligated rats except those operated on with the largest style: They were then visible in 80% of the animals. β-Glucuronidase activity was significantly increased above control values in the renal cortical homogenates of all ligated animals.

Discussion. The histo- and cytopathology of the renal cortex after 10 days of ischemia correspond to our previous observations (3, 5). The level of renin activity found in the control renal cortex by radioimmunoassay is within normal limits. With this technique, depending on the method of homogenization and the purity of the fraction, results have varied between 500 ng angiotensin I/mg protein/hr (15) to 530 μg angiotensin I/μg protein/hr (16). The fivefold increase in renin activity noted in the ischemic renal cortex is also analogous to previous values obtained by bioassay in the same type of kidney (6–8).

Since renin granules contain hydrolytic enzymes (acid phosphatase, β-glucuronidase,



6. Electron microscopic appearance of fraction 5 from ischemic renal cortex. Note the abundance of dense l less electron-dense granules (g_2). Mitochondrion (m) (× 11305).

ABLE II. Effect of Various Degrees of Aortic Constriction on the Protein Content and β-RONIDASE Activity of Renal Cortical Homogenates and on Kidney Weight, Heart Weight, and Final Body Weight

	Style[a]						
p	No.	Diameter (mm)	Protein (mg/g wet wt)	β-Glucuronidase (arbitrary units/g wet wt)	Final body weight (g)	Left (ischemic) kidney weight (mg/100 g body wt)	Heart weight (mg/100 g body wt)
	—	—	91.80	1220.0	236.00	396.00	319.00
			±2.00	±119.0	±8.57	±81.20	±42.30
	26	0.180	72.70[b]	1323.00[b]	143.60[b]	267.00[b]	473.00[b]
			±3.60	±125.00	±3.91	±24.00	±22.00
	30	0.500	59.40[b]	2950.00[b]	131.70[b]	244.00[b]	481.00[b]
			±1.50	±146.00	±3.12	±10.00	±20.00
	18	0.740	62.10[b]	2801.00[b]	188.00[b]	372.00	404.00
			±1.40	±263.00	±9.69	±70.00	±30.00

rtial constriction of aorta using styles of various diameters was performed on the 1st day of the experiment. imals were killed on the 10th day.
gnificant ($P < 0.001$) in comparison with the unoperated control.

ryl sulfatase) (17) and in view of their nously augmented number in the newly d juxtaglomerular cells of the ischemic y (3, 5), the increase of β-glucuronidase, of cathepsin D, found in the ischemic cortex, could be localized either in juxnerular cells or in renal cortical tubular The first results obtained by differential ifugation of the renal cortex indicated renin granules were localized in the mitochondrial fraction together with mes (18–20) in rat, pig, and rabbit kid- Further work, particularly with the inction of discontinuous sucrose density nt centrifugation, showed that lyso- and renin granules are in fact localized ctions of different density in dogs (9), rats (15), and rabbits (13). It has also been demonstrated that renin granules, although they contain hydrolytic enzymes, do not behave like lysosomes (17). The present results confirm that renin granules and lysosomes of the control renal cortex behave differently as regards their localization in fractions of dissimilar density. With a technique identical to the present one, Morimoto *et al.* (9) showed that, in the dog, the top layer, as judged from the distribution pattern of enzymatic activity, was made up of the bulk of rough endoplasmic reticulum and of lysosomes, the middle layer was mostly comprised of mitochondria, and the bottom layer of renin granules. In the rat, isopycnic centrifugation of the heavy granule fraction (obtained by differential cen-

trifugation of the renal cortex) also yielded analogous results (15): Lysosomes were in the top layer (density = 1165), mitochondria in the middle (density = 1175), and renin granules at the bottom (density = 1200). We have also obtained analogous results with the ischemic renal cortex, although here the density of the top layer was lower and renin granules were more dispersed in fractions 4, 5, and 6. These differences are most probably due to the changes in shape, size, and density of lysosomes and renin granules, produced by severe ischemia (3, 5). Since juxtaglomerular cells contain only a very small number of lysosomes (21), our results indicate that the increased hydrolytic enzyme activity of the ischemic kidney is attributable to changes occurring in cortical tubular cell lysosomes, most probably of the proximal convoluted tubular cells because of their concentration of lysosomes (22). Whether these increased lysosomal enzyme activities are located either in the outer or inner cortical tubular cells or in both remains to be determined. Although we did not undertake any quantitative study, the impression was gained that there was no striking increase in the number of lysosomes in the atrophic cortical tubular cells after 10 days of ischemia. It must be emphasized that, at this time period, there are no autophagic vacuoles and no degenerating or necrotic cells in the cortical tubules.

From the data in Table II, it can be deduced that very slight renal ischemia leading to minimal atrophy still produces a significant increment in β-glucuronidase activity. This indicates that, at least in the rat, and probably in other species as well, one cannot induce even mild atrophy of the renal cortex without setting into motion a great rise in hydrolytic enzyme activity in cortical tubular cells. In view of the report indicating that injection of renal lysosomal products may induce angionecrosis and increased vascular permeability (23), experiments are now in progress to find out if, during renal hypertension, lysosomal enzymes of renal origin are present in the systemic circulation.

Severe starvation does not enhance the activity of renal lysosomal enzymes (24). Hence, the intense loss of weight occurring in rats bearing an ischemic kidney (3, 5) is not the cause of the presently found increment of renal lysosomal enzyme activity.

Since cathepsin D is able to split the leu-leu bound of angiotensinogen and thus produce angiotensin I during incubation at acid pH (25), the results of bioassay or radioimmunoassay of renin activity in ischemic kidneys must be interpreted with caution, if the incubation is done at low pH.

Numerous reports have revealed that the activity of certain hydrolytic enzymes is augmented in the urine in case of parenchymal renal damage, both in man (26–30) and in experimental animals (31–33). In controlled studies on the rat, the increased urinary excretion of hydrolytic enzymes has been correlated with degeneration leading to necrosis of cells from the proximal convoluted tubules (31, 33). Apart from the well-known effect of testosterone on mouse kidney where an increase in lysosomal enzyme activity can be detected not only in the urine but also in proximal convoluted tubular cells (34), enhanced hydrolytic enzyme activity (cathepsin and β-glucuronidase) has only been noted in nephrosclerotic rat kidneys following the administration of desoxycorticosterone and NaCl (35) and in spontaneously hypertensive rats with or without NaCl supplements, where an increase in the renal activities, not only of these two enzymes but also of those of RNAse, DNAse, and β-*N*-acetylglucosaminidase, has been observed (36). In both cases, the renin activity of the kidney was decreased. These increased hydrolytic enzyme activities have been tentatively ascribed to vascular changes produced in the kidney by hypertension (35, 36). In view of the present results, they are probably localized in renal cortical tubular cells where zones of atrophy alternating with zones of hyperplasia and hypertrophy are characteristic of nephrosclerosis.

Summary. In rats, severe but partial ligation of the aorta between the renal arteries induces striking changes in the left, ischemic renal cortex: simple atrophy of the outer cortex, and atrophy with hyperplasia and polyploidy of the inner cortical tubular cells. In the ischemic renal cortex, there is a significant increase not only of renin activity but also of β-glucuronidase, of cathepsin D and, to a much lesser degree, of acid phosphatase. The results of isopycnic centrifugation indicate

hat these enhanced hydrolytic enzyme activities are localized in renal cortical tubular cell ysosomes. Since these changes occur even vith minimal atrophy of the kidney, they nust accompany all cases of hypertension of enal origin.

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Prostaglandin Cytoprotection Does Not Involve Interference with Aspirin Absorp (40631)

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In 1976 Robert (1) demonstrated that prostaglandins in nonantisecretory doses protect the gastric mucosa against lesions produced by a variety of agents. He referred to this as the cytoprotective effect of prostaglandins. This finding has been confirmed by other laboratories (2–5), but the mechanism by which prostaglandins provide cytoprotection remains an enigma. Miller and Jacobson (6) recently reviewed the evidence for and against various postulated mechanisms. A number of investigators have shown that prostaglandins inhibit disruption of the gastric mucosal barrier to acid back diffusion by topical barrier breakers (7–9). Bommelaer and Guth (9) suggested that this protection might be due to interference with absorption of the barrier breaker. However, they found that although prostaglandin protected against barrier disruption by topical aspirin, the salicylate content of the gastric mucosa, 1 hr after aspirin administration was the same in prostaglandin-treated and control animals. Nevertheless, the question of a possible effect of prostaglandin on aspirin absorption at a shorter time after aspirin instillation remained. This study was designed to answer that question.

Materials and Methods. Male Sprague–Dawley rats weighing between 150 and 200 g were fasted for 48 hr but allowed water *ad libitum.* Under ether anesthesia the abdomen was opened and the pylorus ligated. Groups of six rats were then injected with either 20 μg/kg 16,16-dimethylprostaglandin E_2 ($dmPGE_2$) s.c. or NaCl (control) s.c. One-half hour after the injection, 40 m*M* acetylsalicylic acid (ASA) in 150 m*M* HCl, 1 ml per 100 g body wt, was instilled into the stomach via orogastric intubation.

Ten, thirty, and sixty minutes after the ASA instillation, the stomach of rats in one $dmPGE_2$ group and one NaCl group were removed. In addition, 1 cm of small bowel 1.5 cm distal to the pylorus, diaphrag aortic blood were obtained before kill rats. The stomachs were opened alo greater curvature, washed in saline, sions scored according to size and n petechiae were given a score of 1, e less than 1 mm 2, 1–2 mm 3, 2–4 mm over 4 mm 5. The stomachs were ther cally divided into forestomach, corpu antrum and the corpus and antral r scraped off. The forestomach, corpus n antral mucosa, small bowel, and diap were weighed, placed in 1 ml perchlor and frozen for salicylate content dete tion. The blood was placed in oxalate, fuged, and the plasma removed for sal determination. Salicylate determi were performed by the Saltzman meth using an Aminco–Bowman spectroflu ter set at 310 n*M* for excitation and 4 for emission. Histologic sections of and antral tissue remaining after n scraping revealed mucosa down to th cularis mucosae had been removed.

Results. Mucosal lesions were prese marily in the corpus of the stomach. animals also had antral lesions but no forestomach lesions. Lesion scores a salicylate concentration of corpus n antral mucosa, and forestomach at th intervals studied are presented in T Lesions were present as early as 10 mi the instillation of ASA and the mean score was lower in the $dmPGE_2$-treat This difference was not significant. W crease in duration of exposure to ASA was an increase in mucosal damage. scores continued to be lower in the dm treated rats and the difference was cally significant at both 30 and 60 mi 0.05, unpaired *t* test).

Gastric salicylate concentration wa est in the forestomach and slightly hi the antral mucosa than the corpus

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I). The concentrations were in the ange at 30 min as at 10 min in all areas, l slightly at 60 min in the corpus and mucosa. In all areas at all times there no significant differences between E_2-treated and control rats. This was so in the corpus mucosa in spite of the cally significant difference in lesion at 30 and 60 min.

:ylate concentrations in small bowel, agm, and plasma are presented in Ta- The concentrations were in the same in these three groups, were much lower 1 the stomach, and, except for a slight plasma concentration, did not change ime. Again there was no significant nce between $dmPGE_2$-treated and con- imals in any group at any time.

ussion. The significant difference in nduced lesions between rats receiving E_2 and saline supports the previously ıstrated cytoprotective effect of this glandin analog (1–5). Although an an- tory dose of $dmPGE_2$ was adminis- the ASA was instilled in 0.15 *M* HCl protective effect could not be due to

I. Lesion Scores and Gastric Salicylate :entration (Mean ± SE) after Different ; of Exposure to Intragastric Acetylsali- Acid in Pylorus-Ligated Rats[a]

	10 min	30 min	60 min
ıcore			
	6.2	19.3	33.2
	±2.2	±2.0	±8.3
$3E_2$	2.6	7.3*	11.2*
	±1.5	±3.6	±5.3
te concentra- ion (nmol/g) us mucosa			
Cl	2.43	2.45	1.97
	±0.48	±0.43	±0.28
PGE_2	2.35	2.62	1.82
	±0.16	±0.41	±0.19
ıl mucosa			
Cl	2.43	3.79	1.69
	±0.47	±0.60	±0.33
PGE_2	3.24	3.55	3.05
	±0.42	±0.55	±0.83
ıtomach			
Cl	4.91	5.52	4.85
	±0.94	±1.02	±0.67
PGE_2	5.05	5.98	5.03
	±1.16	±1.33	±0.69

ere were six rats in each group. An asterisk indi- sults were significantly different from control at $P < 0.05$ (unpaired t test).

TABLE II. Salicylate Concentration (nmole/g, mean ± SE) in Various Tissues at Different Times after Intragastric Acetylsalicylic Acid Administration in Pylorus-Ligated Rats[a]

	10 min	30 min	60 min
Small bowel			
NaCl	0.44	0.46	0.30
	±0.13	±0.13	±0.4
$dmPGE_2$	0.28	0.41	0.50
	±0.12	±0.10	±0.15
Diaphragm			
NaCl	0.63	0.48	0.37
	±0.16	±0.07	±0.09
$dmPGE_2$	0.40	0.63	0.62
	±0.09	±0.06	±0.17
Plasma			
NaCl	0.25	0.30	0.43
	±0.03	±0.05	±0.09
$dmPGE_2$	0.31	0.38	0.40
	±0.05	±0.07	±0.06

[a] There were six rats in each group. None of the values in the $dmPGE_2$-treated rats were significantly different from their control (NaCl) values.

lack of acid (4). In spite of this protective effect, the salicylate concentration in both corpus and antral mucosa was not affected by $dmPGE_2$, thus disproving the hypothesis being tested. The cytoprotective effect of prostaglandin does not involve inhibition of entry of aspirin into the gastric mucosal cells.

Although one would expect a decline in gastric tissue salicylate concentration with time after the intragastric instillation of aspirin, this did not occur as the pylorus was ligated. The stomach mucosa was continuously bathed in an aspirin–acid solution of high concentration throughout the time periods studied. The salicylate concentrations in small bowel and diaphragm were significantly lower than that in the stomach as they reflected blood-borne absorbed aspirin. The concentration in these latter two tissues was approximately the same as in plasma.

Cytoprotection by prostaglandin is a real phenomenon. Robert has demonstrated that local synthesis and release of prostaglandin is an important, natural, gastric cytoprotective mechanism (11). The elucidation of the manner by which prostaglandins exert this effect remains a challenge to investigators in this field.

Summary. Acute gastric mucosal lesions were produced in pylorus-ligated rats by the intragastric instillation of an aspirin–acid solution. Prior administration of 16,16-dimethyl

prostaglandin E_2 s.c. decreased lesion formation but did not affect gastric mucosal salicylate concentration. The cytoprotective effect of prostaglandin does not involve inhibition of entry of aspirin into the gastric mucosal cells.

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m Growth Hormone, Somatomedin and Its Carrier Protein in the Rat: Influence of Age, Sex, and Pregnancy (40632)

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physiology and hormonal regulation wth is a highly complex process, many ; of which remain to be elucidated. ıgh growth hormone (GH), somato- s (SMs), and their carrier protein(s) have been identified as major compo- of the growth-promoting system, little re available concerning the interaction se factors either on the growth of the ıtal–fetal unit or on that of organisms 'etal to adult life. Somatomedins com- ı group of four low-molecular-weight es (SM A, SM C, insulin-like growth [IGF], and multiplication stimulating y), the biological activities of which are ependent (1, 2) and appear to be mod- by circulating CP concentrations (3– gain further understanding about the ı-promoting system, we have examined anges in serum levels of GH, IGF, and in pregnant rats during normal gesta- nd in growing rats from fetal through life. Since prostaglandins (PGs) have hown to act at both the hypothalamic ituitary levels to stimulate GH release), it seemed reasonable that their rise ; gestation might contribute to the mod- ı of the growth-promoting system both maternal and fetal circulations. There- he present study also reports the influ- of the PG synthetase inhibitor, indo- cin, on serum levels of PGs, GH, IGF, s CP in pregnant rats and their off- .

erial and methods. Sprague–Dawley ere housed in an air-conditioned room controlled conditions of light (0600– and temperature (22°). Purina labora- tory chow and water were administered *ad libitum* to adult rats, while pups received mother's milk. Adult male (n = 18) and female (n = 7) rats were housed separately in groups of 4–5 animals per cage; pups (10–12/ dam) stayed with their mothers in individual cages until weaned at Day 20. After weaning, pups were separated according to sex and 4–5 were housed per cage. All animals were weighed prior to each bleeding and blood samples drawn under light ether anesthesia from all except fetal and neonatal animals between 8 and 11 AM. Blood was obtained by decapitation from fetuses on the 20th day of gestation (n = 50), and from neonates (birth to 3 days) at 1 hr, 1 day, and 3 days after birth (n = 20, 18 and 20 pups, respectively). Blood samples from 4 to 5 fetuses, or neonates, were pooled for subsequent assays, while blood sampling was performed by tail artery catheterization approximately weekly (male), or monthly or bimonthly (female), from ages 14 through 120 days. Eleven pregnant rats were studied from the 4th to the 20th day of pregnancy; 6 were untreated while 5 received indomethacin (Merck, Sharpe, and Dohme) (10 mg/kg body wt i.m. twice a day) beginning on the 2nd gestational day. Blood samples were drawn from the tail artery on the 4th, 13th, and 20th day of gestation, and from the uterine vein immediately before the removal of the fetuses under light ether anesthesia on Day 20.

Blood samples obtained from all animals were centrifuged immediately and the sera kept frozen at −20° until assayed for GH by radioimmunoassay (RIA) (11), and IGF and its CP by competitive protein binding (CPB) assays (12, 13). While it is generally agreed that considerable biological, and probably structural, similarity exists between various SMs, rat SM appears to be a basic peptide most closely resembling SMs C and IGF I

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(14). Moreover, it has been shown that SM MSA (multiplication stimulating activity), like SM C, cross-reacts both with IGF I and (to a lesser extent) II in the CPB assay (15, 16) and is detectable in rat plasma by RIA (17). While the CPB assay measures peptides which cross-react with both IGF I and II, it appears that the absolute rat SM levels, herein expressed as IGF reactivity, would be underestimated by this assay since it has been shown that CP has an affinity for IGF I which is approximately only 10% that for IGF II (18). Despite the known limitation in the specificity of the CPB assay, it is felt that it provides a useful approximation of the changes in rat SM levels which have been quantitated in terms of μU/ml relative to an IGF standard, the potency of which has been determined by its stimulation of glucose oxidation in the rat epidymal fat pad bioassay. Samples of maternal and fetal blood used for PG assays were immediately placed in tubes containing 2% EDTA and 10 μg aspirin to inhibit further PG synthesis by platelets *in vitro* during centrifugation. Specific RIAs were employed to measure the principal metabolite of PGF_{2_α} (13,14-dihydro-15-keto-PGF_{2_α}) (PGF M) in maternal plasma and PGE_2 in fetal plasma (19, 20). For statistical analysis, either the paired or unpair Student's *t* test was used as indicated.

Results. A. Growth and the growth-promoting system in growing rats. Consistent weight gain, from 5.0 ± 0.03 g (mean ± SEM) at birth to 424 ± 4 g at 120 days, was observed throughout the experiment. However, the slope of the curve for body weight varied at different ages (Fig. 1). Thus, the initial weight gain (3.6 g/day) between Days 3 and 28, reached its highest rate (6.0 g/day) between Days 28 and 55, then declined slightly (4.9 g/day) between Days 55 and 79, and was minimal (1.4 g/day) between Days 79 and 120. However, the fractional growth velocity in growing rats progressively declined from the birth on (data not shown) in compliance with natural process of growth. The pattern in weight gain was not significantly different in male and female animals during any of the time periods studied.

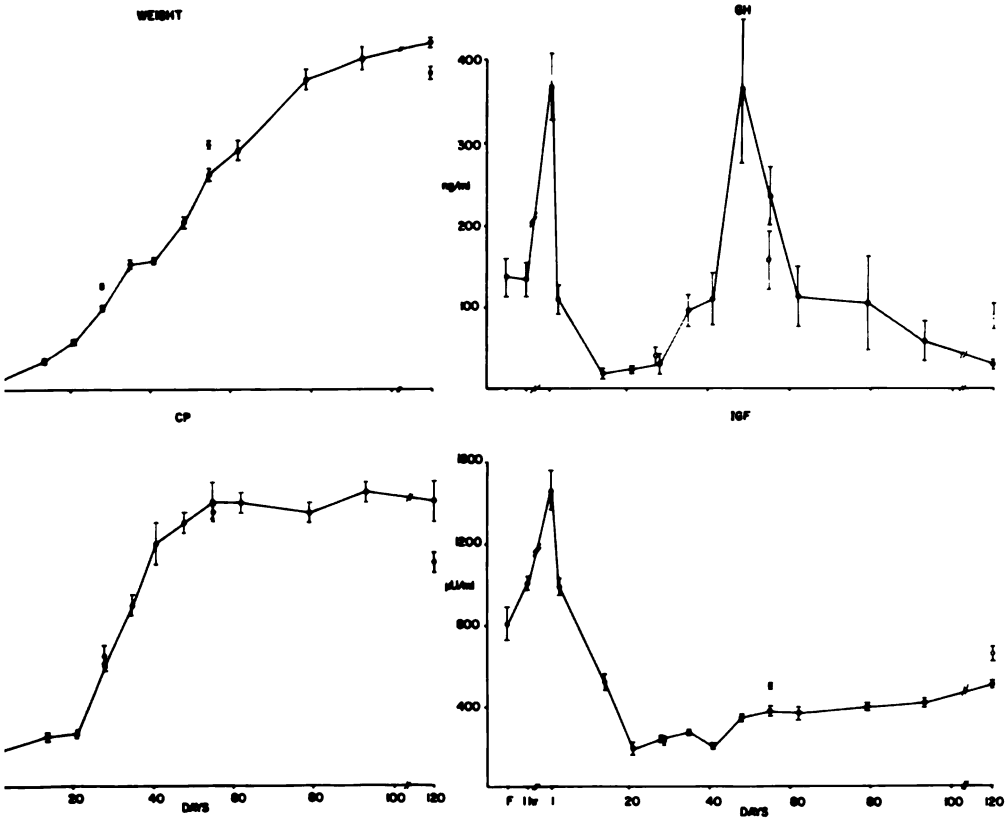
Despite the well-known broad range of GH concentrations in rats, the mean GH levels appeared to be age related (Fig. 1). Thus, the moderately high GH concentration in fetus (137 ± 21 ng/ml) rose almost three on the first day of life (371 ± 37 ng/ml, 0.001), decreased between Days 14 an and reached a second peak at 48 days ± 97 ng/ml) before decreasing to the level (30 ± 4 ng/ml) at 120 days.

During the first month of life the ch in serum IGF levels followed those in concentration ($r = 0.901$; $P < 0.001$). A fetal serum level of 812 ± 164 μU/ml significantly ($P < 0.001$) on the first d life to 2108 ± 26 μU/ml. Thereafter, s IGF rapidly declined to a nadir of 189 μU/ml at Day 21, and then rose gradua a level of 512 ± 12 μU/ml on Day 120 1).

The pattern of IGF CP concentratio sembled that of weight gain rather than of serum GH and IGF (Fig. 1). The CP in fetal (0.28 ± 0.01 mg/ml) and neo (0.36 ± 0.01 mg/ml, Day 3) circulations very low, but doubled by Day 21. There the mean CP level demonstrated a pro sive rise until Day 41 (2.4 ± 0.2 mg/ml) then remained at a plateau (range, 2.6 ± to 2.9 ± 0.1 mg/ml) from 55 until 120 No significant differences were noted i rum GH, IGF, or CP levels between and female rats at any age.

B. Growth-promoting system in pre rats. The body weights of controls (50 g) and indomethacin-treated (528 ± pregnant animals were virtually identi term, as were those of their offspring 0.1 and 5.1 ± 0.2 g, respectively). The of PGF M in the systemic circulatio pregnant controls demonstrated a signi ($P < 0.01$) rise from the 4th (152 ± 2 ml) to the 13th day (256 ± 49 pg/ml) remained elevated on the 20th day of tion (235 ± 29 pg/ml). The administrati indomethacin significantly ($P < 0.0$ duced the levels of PGF M in systemi culation of pregnant rats at all time p studied (Fig. 2).

While serum GH levels were similar in control and indomethacin-treated nant rats on the 4th and 13th days of tion, by the 20th day the mean GH co tration was almost 50% less in the indo acin-treated group than in the control rum IGF levels both in the control a



Body weight and serum GH, IGF, and CP levels in rats. Results are expressed as mean ± SEM. Groups) and neonatal rats include the animals of both sexes (◒). At age 20 days male (●) and female (○) rats were l; no statistically significant sex-related differences in any component of the growth-promoting system were

ıacin-treated rats exhibited a gradual throughout the gestational period, ɔ0 ± 11 to 128 ± 3 μU/ml in controls, ɔm 308 ± 6 to 193 ± 16 μU/ml in ɪthacin-treated animals (4th and 20th ɨpectively). In contrast to reduced GH ɪrations, the mean IGF in indometh-ɜated rats was slightly but significantly).01) higher than in control animals ıout gestation. A dramatic and highly ant ($P < 0.001$) drop in IGF CP con-on was observed in all pregnant ani-ɾith and without indomethacin treat-he slopes of the decrease in CP from to 13 were steep and parallel, drop-ɔm 2.54 ± 0.1 to 0.29 ± 0.11 mg/ml in ɨ, and from 2.43 ± 0.2 to 0.25 ± 0.11 in indomethacin-treated rats.

ɜ control animals the PGF M concen-ɪn the uterine vein on Day 20 (386 ± 54 pg/ml) was significantly ($P < 0.001$) higher than in the systemic circulation (Fig. 3). Indomethacin administration caused a striking reduction ($P < 0.005$) in PGF M concentration both in the systemic and uterine circulation (99 ± 5 and 60 ± 10 pg/ml, respectively) on the 20th gestational day. Similarly, the plasma PGE_2 level in the fetuses of indomethacin-treated mothers (398 ± 152 pg/ml) was significantly ($P < 0.01$) lower than in those of the control mothers (913 ± 42 pg/ml). The administration of indomethacin to pregnant rats caused a significant decrease not only in their plasma PG concentrations, but in serum GH levels as well. The latter were reduced both in the systemic circulation (89 ± 5 vs 50 ± 5 ng/ml, $P < 0.01$) and in the uterine vein (89 ± 8 vs 59 ± 5 ng, $P < 0.01$). The fetal GH concentration in the indomethacin-treated rats (83

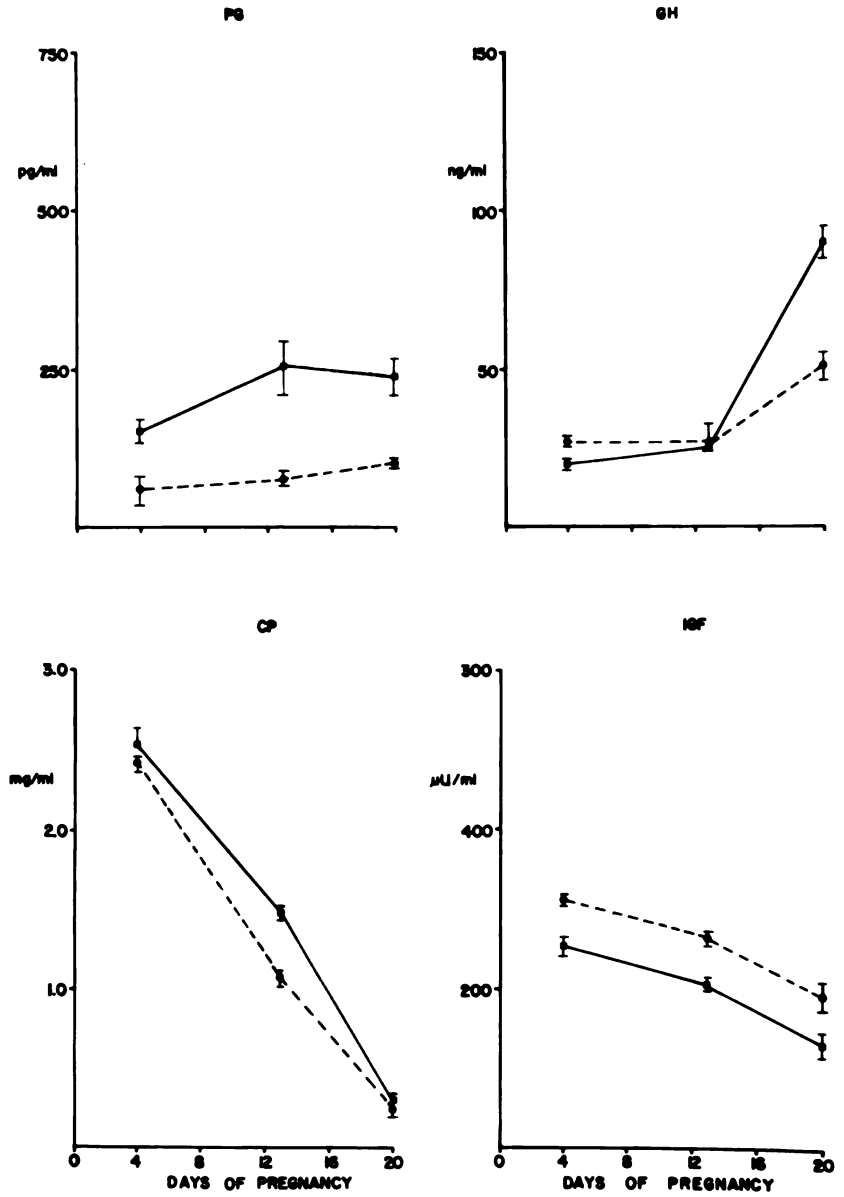


FIG. 2. Plasma PG (PGF M) and serum GH, IGF, and CP levels in control (■——■) and indomethacin-t (●--●) pregnant rats. Indomethacin (10 mg/kg) was injected i.m. twice a day starting on the second gesta day. Results are expressed as mean ± SEM.

± 7 ng/ml) was also significantly lower ($P < 0.05$) than in fetuses of the control group (137 ± 21 ng/ml). The levels of IGF in both maternal systemic circulation and uterine vein of indomethacin-treated rats were significantly higher ($P < 0.001$) than those of control animals. The fetal IGF concentrations, however, were found to be similar both in the control group (812 ± 64 µU/ml) the indomethacin-treated animals (726 µU/ml). The mean CP concentrations i fetal, maternal systemic circulation, ar the uterine vein were virtually identic both groups.

Discussion. The regulation of growth i pendent upon a highly complex system v

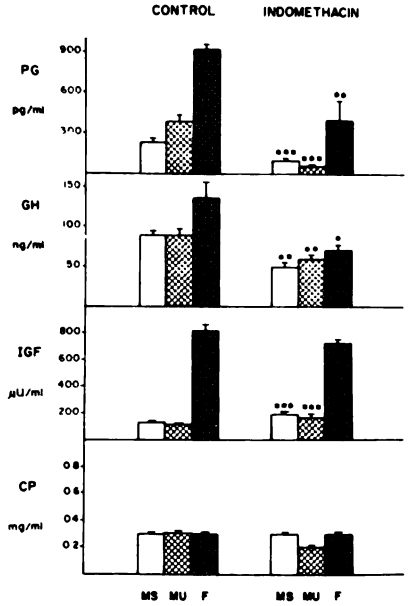


FIG. 3. Plasma PGF M (maternal) and PGE_2 (fetal) and serum GH, IGF, and CP levels in control and indomethacin-treated pregnant rats and their offspring on the 20th gestational day. Values given for the maternal systemic circulation (MS), maternal uterine vein (MU), and fetal circulation (F) are expressed as mean ± SEM. *P* values vs control: * <0.05, ** <0.01, and *** <0.001.

involves the interaction of GH, SMs, and SM carrier proteins. Naturally, GH has a central role in this process by inducing the synthesis and release of SMs which, in turn, mediate many of the effects of GH on peripheral cells (1, 2, 21, 22). Moreover, the biological activity of the SMs appears to be regulated in part by circulating levels of their CPs (3–8, 23, 24). Although many of the components of the growth-promoting system have been identified, the factors which govern the release and action of the interacting substances remain unclear. In this study, we have examined the influence of several factors on the growth-promoting system in the rat. This animal was selected for our studies because in contrast to the human, the rat continues to grow throughout life.

There are two periods during the life of young rats in which serum GH levels appear to be closely related to the rate of growth. The first is during the late fetal and early neonatal life when serum GH is markedly elevated, a finding long recognized both in humans (26) and in rats (27). The importance of elevated GH levels in newborns is emphasized by the observation of marked growth retardation following hypophysectomy at this age (28). The present data, in agreement with these of Ojeda and Jameson (25), demonstrate that high postnatal GH concentrations coincide with a sevenfold increase (from 5 to 36 g) in body weight from birth to age 14 days. Factors regulating the pattern of GH secretion in early infancy are still largely unknown. Although a causative role of estrogens at this age has been suggested, it has also been shown that estradiol treatment does not alter GH synthesis in overiectomized rats (29). Walker *et al.* (30) have demonstrated a highly significant inverse correlation between the hypothalamic somatostatin and serum GH concentrations in neonatal rats, and have emphasized a physiological role in the postnatal development of the hypothalamic regulatory mechanism for pituitary GH release.

The second peak in serum GH concentration occurs prior to sexual maturation (25), coincident with an alteration in growth rate. Our data indicate that the rate of weight gain in male animals (initially 3.6 g/day) almost doubled from Days 28 to 55 (6.0 g/day). Although the rat does not exhibit as marked a "pubertal growth spurt" as the human, Ojeda and Jameson (25) have also found that plasma GH concentrations increase in female rats prior to puberty and are associated with an increase in the slope of the curve for body weight. The increase in GH concentrations during puberty may be related to the influence of the sex steroids which are known to stimulate the release of GH (34, 35).

In contrast to many reports of low SM activity in cord blood of human beings (36–38) and in neonatal rats (39), we have found high levels of the somatomedin IGF in the circulation of fetal and newborn rats. The initial changes in serum IGF follow the changes in GH concentration. As with GH, the elevated level of IGF rose further on the first postnatal day and then decreased to its lowest point by Day 21, following the nadir in serum GH. Thereafter, the IGF concentration demonstrated a continuous but gradual increase to Day 120, departing from the pattern of GH secretion which rose to a second peak at Day 48. Serum IGF CP demonstrated

an entirely different profile. Extremely low in early neonatal circulation, the level of IGF CP rose slowly by the 21st day, exhibited an accelerated rise between Days 21 and 41, and reached a plateau on the 55th day. Although there was no apparent correlation between IGF CP and either GH or IGF levels, there was a striking similarity between the changes in CP levels and the pattern of weight gain.

Pregnancy, with its multihormonal changes, induces new factors into the regulation of the growth-promoting system. Growth hormone secretion and the amplitude of individual pulses in pregnant rats have been shown to be increased on gestational Days 18–21 (40). We have also observed a significant rise in GH levels in control pregnant animals on the 20th day of gestation. Nevertheless, despite this obvious rise, the mean maternal GH concentration was far less than that of the offspring. A slight, but significant, decrease in IGF concentration throughout gestation and the unexpectedly high fetal IGF levels pose additional questions and emphasize the differences between the rat and human growth-promoting systems. Thus, in pregnant women, the mean IGF concentration exhibits a tendency to rise while its level in cord blood is barely half that in the maternal circulation; in contrast, the decrease of IGF CP level in pregnant rats was similar to that observed in humans (41). The physiological significance and possible cause(s) of this reduction in CP concentration during pregnancy are unknown. An identical observation that had been noted in the serum of pregnant women (41) led us to investigate the possibility of a rise in serum-"free" IGF concentration. The data obtained have demonstrated that "free" IGF levels in pregnant women were approximately threefold higher than those in nonpregnant controls (manuscript submitted for publication).

Since PGs have been repeatedly shown to increase GH release (31–33), a significant rise in their concentration during gestation may contribute to the elevation in maternal GH concentration in late pregnancy and to that in the fetal–neonatal circulations. PG measurements in plasma provide only an indirect index of synthesis by various tissues. Nevertheless, the significant decreases in PG concentration at three sites (maternal systemic, maternal uterine, and fetal circulations) attest to the widespread inhibition of PG synthesis induced by indomethacin; the blockage affects all compounds derived from the cyclooxygenase pathway. Lower concentrations of PGs in indomethacin-treated pregnant rats and their offspring were accompanied by lower GH levels as compared to controls. Surprisingly, the IGF levels in maternal circulation in indomethacin-treated animals were slightly, but significantly, higher than in the control group. Furthermore, in spite of substantial differences in GH concentration, IGF levels and fetal weights were similar in indomethacin-treated and control animals. This apparent lack of positive correlation between serum GH and IGF levels in indomethacin-treated rats suggests that substances other than GH may govern SM production in pregnant rats. Thus, Daughaday and his colleagues (42, 43) have recently demonstrated that in rats hypophysectomy performed on the 14th gestational day was not accompanied by decrease in SM concentration until after delivery. They have concluded, therefore, that SM concentration in pregnant rats was probably maintained by placental somatomammotropin.

Summary. The changes in body weight and serum levels of growth hormone (GH), somatomedin insulin-like growth factor (IGF), and its carrier protein (CP) have been followed in rats from fetal to adult life. The weight gain was maximal (6.0 g/day) between Days 28 and 55 and minimal (1.4 g/day) between Days 79 and 120. Two distinct peaks of GH concentration have been found in normally growing animals. The first (mean ± SEM) was observed on the first day of life (371 ± 37 ng/ml) while the second peak (365 ± 97 ng/ml) coincided with an acceleration in growth rate beginning Day 40. An unexpectedly high fetal IGF level (812 ± 164 μU/ml) rapidly declined to a nadir (189 ± 26 μU/ml) at Day 21, and then rose gradually to a level of 572 ± 12 μU/ml on Day 120. The pattern of IGF CP concentration resembled that of weight gain rather than those of serum GH and IGF. The sex of animal appeared to have no influence on any component of growth-promoting system.

The administration of indomethacin (10 mg/kg) to pregnant animals inhibited the

elevation not only of prostaglandins in maternal (99 ± 5 vs 235 ± 29 pg/ml) and fetal (398 ± 152 vs 913 ± 42 pg/ml) circulations, but also of GH (50 ± 5 vs 89 ± 5 ng/ml, and 83 ± 7 vs 137 ± 21 ng/ml, respectively).

In contrast, serum IGF levels in indomethacin-treated pregnant rats were slightly but significantly elevated above those found in control animals throughout gestation. An unexplained dramatic decrease in IGF CP concentrations was observed in both groups of pregnant rats.

This study of the growth-promoting system in growing rats demonstrates its complexity and age dependency. Pregnancy is accompanied by significant and specific changes in all major components of the growth-promoting system. Some of these alterations may be related to elevated levels of PGs while the reasons for the others are still unknown.

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Important Variables in Granulocyte Chemiluminescence (40633)

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st report of granulocyte chemilumi- (CL) during phagocytosis was by d co-workers (1). The light produc- related with metabolic activation curs during phagocytosis. Chemilu- ce has been increasingly used as a follow granulocyte metabolic activ- ated with microbicidal events (2, 3). s been used as a measure of phago- id opsonins (4–6). Techniques of CL ive varied widely, including differ- temperature, granulocyte isolation es, and cell media. This report ex- ome of these variables in order to nore optimal conditions for future es.

als and methods. Granulocyte prepa- Blood was obtained from healthy dogs that had been dewormed, and ed against hepatitis and distemper. od was anticoagulated with either 10 U/ml of blood) or EDTA (0.4 ml DTA/16 ml of blood). Red blood Cs) were sedimented by adding 3 ml dextran in saline (average MW to every 10 ml of anticoagulated fter the RBCs had settled out for 30– ne supernatant solution was removed rifuged (750 rpm, 66*g* for 10 min) to : white blood cells (WBCs) and re- BCs. The cell pellet was suspended of cold NH_4Cl RBC lysing solution 8.32 g/liter, $NaHCO_3$ 0.84 g/liter, 3.2 mg/liter) for 10 min at 4° (7). In eriments the hypotonic lysis proce- Goldstein *et al.* (8) was used to re- ICs. RBCs were hypotonically lysed nding the cell pellet in 8 ml of 0.9% lding 24 ml of distilled water for 20 then adding 8 ml of 3.6% NaCl to isotonic conditions. After the RBC cedure the remaining WBCs were wice (500 rpm, 30*g* for 10 min) with a, resuspended, and counted. Large r aggregates were removed by pour- ing the WBC suspension through a fine-m stainless-steel filter (0.2-mm openings). W counts were determined with a Cou counter (Model Zf), and differential cou were done by microscopic evaluation Wright-stained smears. On the basis of WBC count and differential the volum the cell suspension was adjusted to give desired granulocyte count.

Chemiluminescence measurement. Gra locyte chemiluminescence was measured v a specifically designed chemiluminesce spectrometer which had temperature cont magnetic mixers, and rubber injection p to permit the addition of reagents to reaction vial when positioned in front of photomultiplier tube (9). The disposable action vials were made of polyethylene pla (Minivials, Rochester Scientific Co., Ro ester, N.Y.). During each CL run the sam were maintained at 37° with continuous n ing. Emitted light was measured and recor as counts per minute (cpm). Most CL exp iments were assayed for 10 min after addi of the stimulus. Background counts of stimulated granulocytes were determined mediately before adding the CL stimu The granulocytes (5×10^6) were stimula in all CL experiments with 2 mg of opsoni zymosan. Zymosan was prepared by suspe ing the particles in saline, heating in a boi waterbath for 60 min, and washing th times with saline. Zymosan was opsonized incubating 2 mg of zymosan with 0.1 m autologous serum at 37° for 10 min. experiments were run in triplicate unless erwise stated, using granulocytes from same donor. In most cases the experim were repeated at least once using granuloc from different donors. Consistent differe were found.

Chemicals and cell media. All chemi were of reagent grade. The following media were used: Hanks' balanced salt s tion (HBSS; pH 7.4; Gibco, Grand Isl

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N.Y.), Earles' balanced salt solution (EBSS; pH 6.8; ISI, Cary, Ill.) Ringers' solution (Baxter, Travenol), medium 199 with Earles' salts (M199; pH 7.4; Gibco), minimal essential medium with Earles' salts (MEM; pH 7.6; Microbiological Associates, Bethesda, Md.), and Hepes medium (pH 7.5; Microbiological Associates). Compositions of the cell media are shown in Table I. None of the media contained phenol red in order to avoid color quenching during the CL assay. ACD formula A was obtained from Aminco, Travenol, and disodium ethylenediamine tetraacetate (EDTA) from Fisher Scientific Company.

Results. Temperature of cells during isolation. The temperature of granulocytes during CL assays has a marked effect on the amount of light emitted (9). In the present experiment we evaluated the influence of temperature during the isolation of cells on the CL reaction. Figure 1 illustrates the effect of leukocyte isolation temperature on granulocyte CL responses (temperature of CL assay was 37° for all experiments). The best results were obtained with cells kept at 4° during the isolation procedure with approximately twice as much light produced as cells isolated at 37°.

An important correlary finding was that leukocyte aggregation increased with temperature during granulocyte isolation. Cells prepared at 37° had many large aggregates grossly visible, while few were found at 27° and none were visible at 4°. The decreased CL observed from cells isolated at higher temperatures may have been due to increased aggregation at these temperatures. Maintaining cells at 4°, however, did not completely eliminate aggregation in all experiments. This led to the investigation of other techniques designed to further reduce granulocyte aggregation.

Aggregation. In evaluating procedures for their ability to reduce aggregation we have compared cell preparations after incubating separate aliquots at 4 and 37° for 1 hr. Since incubation at 37° encourages the development of aggregation, those techniques which prevented aggregation at that temperature were considered the most useful for CL studies. Aggregation was determined by a decrease in the total WBC count as measured by a Coulter counter. This served as a meas-

TABLE I. COMPOSITION OF CELL MEDIA (g/liter)

	Ringers	HBSS	Earles	Hepes	Medium 199	MEM
$CaCl_2$	0.25	0.14	0.20	0.20	0.20	0.20
KCl	0.42	0.40	0.40	0.40	0.40	0.40
KH_2PO_4	—	0.06	—	—	—	—
$MgCl_2 \cdot 6H_2O$	—	0.10	—	—	—	—
$MgSO_4 \cdot 7H_2O$	—	0.10	0.20	0.20	0.20	0.20
NaCl	9.00	8.00	6.80	6.80	6.80	6.80
$NaHCO_3$	—	0.35	2.20	—	1.25	2.20
$Na_2HPO_4 \cdot 7H_2O$	—	0.09	—	—	—	—
$NaH_2PO_4 \cdot H_2O$	—	—	0.14	0.14	0.12	0.14
$Fe(NO_3)_3 \cdot 9H_2O$	—	—	—	—	7.2×10^{-4}	—
Glucose	—	1.00	1.00	1.00	1.00	1.00
Hepes[a]	—	—	—	238	—	—
Other components (mg/liter)	—	—	—	—	[b]	[c]

[a] *N*-2-Hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

[b] Adenine sulfate—10.0, adenosinetriphosphate—1.0, adenylic acid—0.2, cholesterol—0.2, deoxyribose—0.5, glutathione—0.05, guanine—0.3, hypoxanthine—0.3, ribose—0.5, sodium acetate—50.0, thymine—0.3, tween 80—20.0, uracil—0.2, xanthine—0.3, DL-α-alanine—50.0, L-arginine—70.0, DL-aspartic acid—60.0, L-cysteine—0.11, L-cystine—20.0, DL-glutamic acid—150.0, L-glutamine—100.0, glycine—50.0, L-histidine—21.88, l-hydroxyproline—10.0, DL-isoleucine—40.0, DL-leucine—120.0, L-lysine—70.0, DL-methionine—30.0, DL-phenylalanine—50.0, L-proline—40.0, DL-serine—50.0, DL-threonine—60.0, DL-tryptophan—20.0, L-tyrosine—40.0, DL-valine—50.0, ascorbic acid—0.05, α-tocopherol phosphate—0.01, D-biotin—0.01, calciferol—0.1, Ca-pantothenate—0.01, choline chloride—0.5, folic acid—0.01, *i*-inositol—0.05, menadione—0.01, niacin—0.025, niacinamide—0.025, *p*-aminobenzoic acid—0.05, pyridoxal—0.025, pyridoxine—0.025, riboflavin—0.01, thiamine—0.01, vitamin A—0.14.

[c] L-Arginine—126.4, L-cystine—24.0, L-glutamine—292.0, L-histidine—41.9, L-isoleucine—52.5, L-leucine—52.4, l-lysine—73.1, l-methionine—14.9, L-phenylalanine—33.0, L-threonine—47.6, L-tryptophan—10.2, L-tyrosine—36.2, L-valine—46.8, D-calcium pantothenate—1.0, choline chloride—1.0, folic acid—1.0, *i*-inositol—2.0, nicotinamide—1.0, pyridoxal—1.0, riboflavin—0.1, thiamine—1.0.

ure of aggregation since aggregated cells are counted as single cells in the Coulter counter. The WBC counts were closely observed to be sure that large aggregates did not occlude the counting chamber and give incorrect counts. Table II shows the effect of Ca^{2+}- and Mg^{2+}-free HBSS when compared with HBSS containing these ions. Also shown are the results obtained when the Ca^{2+}- and Mg^{2+}-free HBSS contained chelating agents (ACD formula A 1.5 ml/100 ml of Ca^{2+}-Mg^{2+}-free HBSS or EDTA 0.125% in Ca^{2+}-Mg^{2+}-free HBSS). Marked aggregation occurred at 37° in HBSS containing Ca^{2+} and Mg^{2+}. There was a slight reduction in aggregation when Ca^{2+}- and Mg^{2+}-free HBSS was used, however, both chelating agents caused a dramatic reduction in aggregation. Gross and microscopic examination of the samples and measurements of light scattering (OD 550 nm) correlated well with the Coulter counter results (data not shown). When heparin (10 U/ml) was used as an anticoagulant, and the same amount was added to HBSS in the isolation and preparation of the granulocytes, the level of aggregation was greater than that found when granulocytes were prepared with Ca^{2+}- and Mg^{2+}-free HBSS (data not shown).

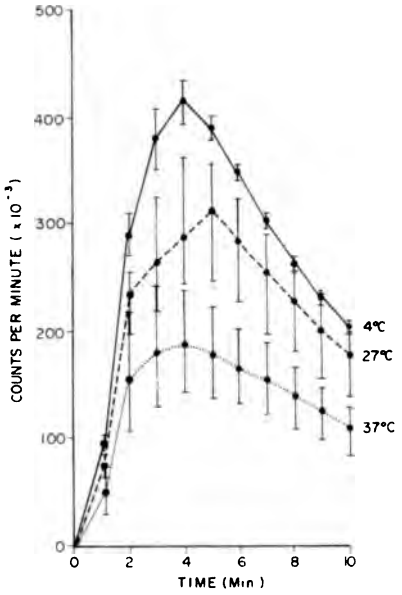

FIG. 1. Granulocytes were isolated and maintained at 4, 27, or 37° until 5 min before the CL assay when they were placed in a 37° water bath. The CL assay was run at 37° with continuous mixing. Opsonized zymosan was used as the CL stimulus. The lines indicate the average of triplicate determinations, and the brackets show the ranges.

Another factor contributing to aggregation was the technique of RBC lysis. In CL assays it is necessary to remove the RBCs because of their light quenching ability (9). Two techniques commonly used are hypotonic lysis (8) and ammonium chloride lysis (7). Table III summarizes the amount of aggregation associated with these procedures. The ammonium chloride method had a degree of aggregation which was similar to hypotonic lysis performed once. When hypotonic lysis was performed twice, however, there was a marked increase in the degree of aggregation. A similar increase in aggregation might have been seen if ammonium chloride lysis had been performed twice; but, in our experience it is rarely necessary to repeat ammonium chloride lysis in order to remove all the RBCs while it is frequently necessary to perform hypotonic lysis twice.

The force of centrifugation was another cause of aggregation. The centrifugation forces listed in the materials and methods section were the minimal values that would

TABLE II. EFFECT OF CALCIUM AND MAGNESIUM IONS ON GRANULOCYTE AGGREGATION[a]

	White blood cell counts per mm^3 using Coulter counter			
	Incubated 1 hr at 4°		Incubated 1 hr at 37°	
Cell media	Average	Range	Average	Range
HBSS	11,129	(11,055–11,194)	6,650	(6,570–6,697)
Ca^{2+}-Mg^{2+} Free HBSS	12,879	(12,822–12,956)	7,860	(7,667–8,195)
Ca^{2+}-Mg^{2+} Free HBSS + ACD	13,037	(12,905–13,263)	10,420	(10,339–10,546)
Ca^{2+}-Mg^{2+} Free HBSS + EDTA	11,105	(11,038–11,201)	10,953	(10,786–11,074)

[a] In this experiment the cell media listed were used for washing the cells following RBC lysis and in the final cell suspension used during incubation. Cells were maintained at 4° during the isolation procedure prior to the final incubations indicated above. All samples were equal aliquots of a single WBC preparation taken through the point of RBC lysis. The values shown are the average and range of triplicate WBC counts using a Coulter counter.

TABLE III. EFFECT OF RBC LYSING PROCEDURE ON GRANULOCYTE AGGREGATION[a]

	White blood cell counts per mm^3 using Coulter counter			
	Incubated 1 hr at 4°		Incubated 1 hr at 37°	
Lysing procedure	Average	Range	Average	Range
NH_4Cl Lysis*	12,199	(12,060–12,342)	10,060	(10,039–10,102)
Hypotonic lysis**				
Performed once	11,646	(11,486–11,807)	9,251	(9,162–9,300)
Performed twice	11,695	(11,574–11,758)	4,573	(4,497–4,622)

[a] All cells were prepared using Ca^{2+}-Mg^{2+}-free HBSS + EDTA 0.125%. Cells were maintained at 4° during the isolation procedure prior to the final incubations indicated above. The average of three determinations is shown.

* Weening *et al.* (7).

** Goldstein *et al.* (8).

completely sediment the granulocytes in a 10-min period. Centrifugation at higher speeds caused an increase in gross and microscopic aggregates that could not be broken up by vigorous vortex mixing.

In an attempt to further reduce cell aggregation, heated and unheated 10% autologous serum was used with HBSS. Neither of these preparations had any effect in reducing aggregation (data not shown).

Calcium and magnesium requirements for optimal chemiluminescence. Granulocytes require Ca^{2+} and Mg^{2+} for optimal light production. Since it was shown above that cells prepared in the absence of these ions were less likely to aggregate, we wanted to determine whether adding these ions immediately prior to a CL assay would allow the cells to produce normal levels of light. Figure 2 shows the CL responses of zymosan-stimulated granulocytes when the cells were suspended in either standard HBSS or in Ca^{2+}- and Mg^{2+}-free HBSS. Very little light was detected in the absence of Ca^{2+} and Mg^{2+}. The results of experiments in which granulocytes were prepared in Ca^{2+}- and Mg^{2+}-free HBSS containing EDTA 0.125% (3.4×10^{-3} *M*) are also shown in Fig. 2. In these experiments excess Ca^{2+} and Mg^{2+} were added immediately before initiating the CL run in order to bind EDTA and permit free Ca^{2+} and Mg^{2+} for granulocytes. The lowest final concentration of Ca^{2+} and Mg^{2+} was obtained by adding: $CaCl_2$, 0.28 g/liter; $MgSO_4 \cdot 7H_2O$, 0.24 g/liter; and $MgCl_2 \cdot 6H_2O$, 0.24 g/liter ($Ca^{2+} = 2.5 \times 10^{-3}$ *M*, $Mg^{2+} = 2.2 \times 10^{-3}$ *M*). The other three concentrations tested were 1.5, 2, and 3 times this level. All four concentrations gave similar levels of CL which exceeded the CL of cells prepared in standard HBSS (Fig. 2).

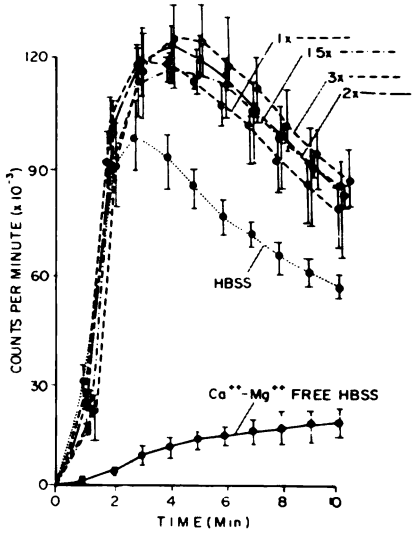

FIG. 2. The CL response of zymosan-stimulated granulocytes in various cell media is shown. The bottom two lines show the difference in CL responses of granulocytes in HBSS and Ca^{2+}- and Mg^{2+}-free HBSS. Granulocytes used in the four top lines were isolated in Ca^{2+}- and Mg^{2+}-free HBSS containing EDTA (0.125%; 3.4×10^{-3} *M*). In the experiment shown by the line marked 1X, Ca^{2+} and Mg^{2+} were added (see text) just prior to the CL assay. In the other experiments 1.5, 2, or 3 times the amount of Ca^{2+} and Mg^{2+} as used in the 1X experiment were added prior to the CL assay. The assay was performed at 37° with continuous mixing. Each line is the average of three observations and the brackets show the range.

Cell media and additives. A variety of cell media (compositions shown in Table I) were examined for their influence on granulocyte CL. Many of these media have been used by other investigators in CL studies. HBSS gave

est CL levels while EBSS was next best
e IV). All of the other media, however,
markedly reduced levels of CL. In order
arify the reasons for these differences
of the components present in these cell
a were individually examined.
e effects of various medium components
anulocyte CL are summarized in Table
ll of the proteins added to HBSS in-
d the CL responses of zymosan-stimu-
cells. In some cases, such as the reac-
containing 2% ovalbumin, almost no
vas detected. The suppressive effect of
roteins did not appear to be due to light
ching since most of the solutions were
less or only faintly yellow. Of the two
o acids tested, tryptophan caused a sig-
nt suppression of CL while tyrosine had
fect. Glucose enhanced the CL response
lls to opsonized zymosan. CL reactions
SS (normally containing 1 g of glucose/
were greater than reactions in HBSS
the glucose omitted. The CL response
urther augmented when the glucose level
ncreased to 2 g/liter. Granulocytes in
S with 4 g of glucose/liter had similar
of CL as cells in HBSS with 2 g of
se/liter. Ascorbic acid and ferrous and
salts all had suppressive effects on the
f zymosan-stimulated granulocytes.

cussion. One of the greatest limitations
e use of granulocyte CL as a research
ique or a clinical assay is its variability.
e of the first experiments in evaluating
actors contributing to this variability, it

E IV. THE INFLUENCE OF CELL MEDIA ON THE LUMINESCENCE OF GRANULOCYTES STIMULATED WITH OPSONIZED ZYMOSAN

Cell media	Chemiluminescence[a] Average (%)	Range (%)
HBSS	100	89–111
EBSS	60	54–65
M199	49	46–53
Hepes	2	1–2
Ringers	1	0–1
MEM	<1	0–1

[a] his table includes data from experiments per-l on different days, but in each experiment HBSS mpared with other cell media. For the purpose of rison CL (total counts per 10-min run minus ound values) was expressed as percentage of The average CL value for HBSS was arbitrarily ered to be 100%. The range of duplicate determi-is shown.

TABLE V. THE INFLUENCE OF ADDITIVES ON THE CHEMILUMINESCENCE OF GRANULOCYTES STIMULATED WITH OPSONIZED ZYMOSAN

Cell media	Chemiluminescence[a] Average (%)	Range (%)
HBSS—No additives	100	89–117
HBSS + additives		
Protein		
Autologous serum 2% (v/v)	76	72–79
Autologous serum 10% (v/v)	51	47–54
Bovine serum albumin 2% (w/v)	29	15–38
Fetal calf serum 2% (v/v)	59	59–60
Fetal calf serum 10% (v/v)	39	38–40
Gelatin 3% (w/v)	45	16–78
Ovalbumin 2% (w/v)	2	2–2
Amino Acids		
Tryptophan 3×10^{-4} *M*	73	64–82
Tyrosine 5×10^{-4} *M*	110	104–117
Glucose (g/liter)		
None	84	80–86
1[b]	100	89–117
2	124	122–125
4	123	117–129
Ascorbic acid 1×10^{-4} *M*	61	58–64
$FeSO_4$ 3.7×10^{-5} *M*	52	46–58
$FeCl_3$ 3.7×10^{-5} *M*	54	41–61

[a] This table includes data from experiments performed on different days, but in each experiment HBSS with and without additives were compared. For the purpose of comparison CL (total counts per 10-min run minus background values) was expressed as percentage of HBSS without additives. The average CL value for HBSS—no additives—was arbitrarily considered to be 100%. The range of triplicate determinations is shown.

[b] HBSS + glucose 1 g/liter is the same as HBSS—no additives.

was found that the temperature at which the cells were isolated significantly influenced the amount of observed CL. Cells prepared at 4° gave much better CL than those prepared at 37° when exposed to opsonized zymosan at 37°. Since increased granulocyte isolation temperature correlated with increased cell aggregation, it appeared to be a likely cause of reduced CL. Aggregated granulocytes would have difficulty phagocytizing a particulate stimulus such as zymosan. Even soluble stimulators of granulocyte CL might be hindered by aggregation. Aggregation could also lead to inhomogeneity of samples and consequently greater variability.

Using the observed potentiation of aggre-

gation at 37°, we examined various techniques to reduce aggregation. Other authors (10, 11) have noted that divalent cation chelators were helpful in reducing granulocyte aggregation. The present study has shown that the addition of EDTA and ACD to Ca^{2+}- and Mg^{2+}-free media caused a significant reduction in aggregation at 37° compared to Ca^{2+}- and Mg^{2+}-free media without chelators. It was possible to obtain high CL responses from cells in these media by adding excess Ca^{2+} and Mg^{2+} to the cells immediately prior to the CL assay. The responses were, in fact, better than those observed from cells prepared with standard HBSS. This improvement was probably due to reduced cell aggregation.

Physical trauma such as that produced by centrifugation and resuspension of cell pellets contributes to granulocyte aggregation. Reducing centrifugation speed and gentle mixing to resuspend cell pellets appeared to be beneficial in reducing granulocyte aggregation. The technique of RBC lysis is another important cause of aggregation. The ammonium chloride method caused less aggregation than the hypotonic lysis of RBCs under conditions which permitted complete lysis of the RBCs. By careful attention to all of the factors that prevent aggregation granulocyte CL should be greater and more reproducible.

Reports of CL studies show a confusing array of cell media ranging from buffered salt solutions containing no glucose, Ca^{2+}, or Mg^{2+} (4) to buffered salts containing proteins, glucose, Ca^{2+}, Mg^{2+}, and complex mixtures of amino acids, vitamins, and other additives (12–14). The present study demonstrates that the more complex media containing multiple amino acids and vitamins gave much lower levels of CL than the buffered salts with glucose, Ca^{2+}, and Mg^{2+}. It was found that proteins in general, tryptophan, ascorbic acid, and ferrous and ferric salts all had a significant suppressive effect on granulocyte CL. Some of these observations appear to contradict the results of Nelson *et al.* (12) who reported augmented CL in the presence of 3% gelatin and 3×10^{-4} *M* tryptophan. They suggested that these substances were serving as substrates for the reactive oxygen intermediates produced by the activated granulocytes. The systems are not identical, however, since these investigators used human granulocytes in a CL assay performed at ambient temperature.

Since glucose is an energy source for CL, it is possible that added glucose might augment the supply from cellular glycogen stores and thereby cause increased CL. This idea was supported by these studies. Although CL was observed without added glucose, the maximum response occurred when the glucose concentration was approximately 2 g/liter. Another, but less likely, possibility was that oxygen intermediates were reacting directly with glucose to produce a chemiluminescent reaction.

This study demonstrates that optimal granulocyte CL occurs when cells are in a buffered salt solution with glucose, Ca^{2+}, and Mg^{2+} without proteins, amino acids, vitamins, and iron salts. Minimizing the physical forces acting on the cells, reducing the temperature of cell isolation to 4°, and the use of Ca^{2+}-Mg^{2+}-free media with added divalent cation chelators are helpful in reducing aggregation and improving CL. These observations may be useful in improving the design of future CL studies.

Summary. A CL system consisting of canine granulocytes stimulated with opsonized zymosan was used to examine factors which lead to variability in light production. Variability of CL response was associated with granulocyte aggregation. Granulocyte aggregation was reduced by: (i) reducing centrifugation and mixing forces used to prepare cell suspensions, (ii) preparing cell suspensions at 4° in Ca^{2+}- and Mg^{2+}-free cell media containing divalant cation chelating agents, and (iii) lysing red blood cells by the ammonium chloride technique.

The type of cell media used during the CL run was another important variable. Solutions containing complex mixtures of amino acids and vitamins gave markedly reduced levels of CL. Tryptophan, ascorbic acid, iron salts, and all proteins tested caused significant suppression of CL. Glucose, Ca^{2+}, and Mg^{2+} in the final reaction mixture were necessary for maximal CL response. The optimal cell media for CL, therefore, was buffered salt solution containing glucose, Ca^{2+}, and Mg^{2+}, but not amino acids, vitamins, proteins, or iron salts.

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Effect of Graded Arterial Occlusion on Ileal Blood Flow Distribution (40634)

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In recent years there has been growing interest in the intestinal microcirculation relative to its ability to regulate blood flow and oxygen delivery. The role of local myogenic and metabolic factors in the regulation of intestinal blood flow has received much attention and support (1, 2). The effects of graded arterial occlusions on blood flow are frequently used to elucidate the mechanism(s) involved in the intrinsic regulation of blood flow. An assumption inherent in previous studies is that the reactive hyperemic response to arterial occlusion occurs uniformally throughout the wall of small intestine (2–4).

While various physiological and pharmacological interventions are known to alter blood flow distribution to the various layers of the intestinal wall the effect of graded arterial occlusion on blood flow distribution has not previously been studied. The present study was designed to examine the effect of graded arterial occlusion (60s, 120s) on blood flow distribution in the small intestine.

Methods. The experimental preparation used in this study is similar, except for minor alterations, to that described in detail in a previous study (3). In brief, six cats previously fasted for 18 hr, were initially anesthetized with 150 mg ketamine HCl. The right femoral vein was cannulated for administering sodium pentobarbital. Systemic arterial pressure was measured with a Statham P23A transducer connected to a femoral artery cannula. A tracheotomy was performed to facilitate breathing and as a means of artificial respiration if the cats failed to breath spontaneously during the experiment. A midline abdominal incision was made, and the greater omentum, lesser omentum, spleen, large intestine, duodenum, jejunum, and a small portion of proximal ileum were extirpated. In some animals the stomach was surgically removed due to excessive distention. Immediately after isolation of the ileal segment and before cannulation of the superior mesenteric artery and vein, heparin (1 mg/kg) was administered intravenously. Body temperature was maintained at 37° by use of an overhead infrared lamp. To minimize evaporation and tissue hydration, all exposed tissue was moistened with saline-soaked gauze and covered with a plastic sheet.

The superior mesenteric (SM) artery was rapidly ligated and cannulated in both distal and proximal directions. The ends of the two cannulas were connected via a Carolina Medical Electronics flow probe with pressure ports. Superior mesenteric arterial pressure was measured through one of the flow probe pressure ports, which was connected to a Statham P23Ac transducer. The signal from the flow probe was used to drive a square-wave electromagnetic flowmeter (Caroline Medical Electronics). A large polyethylene cannula was inserted into the superior mesenteric vein, and venous outflow was passed into a reservoir before being returned to the cat through a jugular vein. The end of the venous outflow cannula was firmly attached to a vertically positioned pulley system. Venous outflow pressure of the intestinal segment was set by adjusting the height of the reservoir and monitored from a T connector located in the venous circuit. Heparinized blood from a freshly sacrificed donor cat was used to prime the extracorporeal venous and arterial circuits. The remaining portion of the mesenteric pedicle was tied off to ensure that venous outflow represented the total organ value. Blood flow distribution within the different layers of the small intestine was determined by use of multiple pulse injections of radiolabeled microspheres (^{85}Sr, ^{169}Yb, and ^{141}Ce, 3M Co., St. Paul, Minn.). The radiolabeled microspheres, with a mean diameter of 15 μm were injected into a rubber port in the mesenteric arterial loop located immediately distal to the flow probe. The microsphere-containing arterial blood entered a mixing chamber before entering the circulation of the small intestine. Blood was with-

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ı for 4 min at a constant rate (0.51 ml/ from a rubber port in the arterial loop ıd immediately distal to the mixing ber. The total counts in the withdrawn was used to calculate absolute blood In general, the ^{141}Ce microspheres (0.2 μCi) were injected into the arterial loop ɛ control state when systemic arterial ıre, superior mesenteric and venous ıres, and intestinal blood flow were in dy state. Ten to fifteen minutes after the injection, the superior mesenteric artery ccluded proximal to the flow probe for :, after which the occlusion was released. ın seconds prior to releasing the occlu- 5 Ci (0.2 ml or approximately 40,000 spheres) of ^{85}Sr microspheres were in- into the occluded arterial loop proxi- o the mixing chamber. After releasing :clusion, blood was withdrawn for 4 min the control condition. Ten to fifteen ɛs after a new steady state was achieved terial loop was occluded for 120 sec and foremention ed procedure was repeated ^{169}Yb microspheres. In four prepara- ^{85}Sr, ^{169}Yb, and ^{141}Ce microspheres injected in random sequence during y-state blood flow in order to determine results were due to differences among icrospheres rather than the experimen- tervention.

er the 4-min blood withdrawal (120-sec ıion) the mucosal–submucosal samples acquired by scraping (3-cm length) the sal–submucosal layers from the remain- ssue with glass microscope slides, and ıderlying muscle layers were cut into 3- ng sections (10 samples). All mucosal– ucosal and muscularis samples were ı in counting tubes and counted in a channel gamma counter (Packard l 3000). Corrections were made for ıp in the gamma energy spectrums. ol and reactive hyperemic blood flows ın each tissue sample (per gram wet wt) calculated from the blood withdrawal WR), total counts/min of arterial blood rawn during each period (BC), and the s/min of each isotope in each tissue e (TC) assuming BF = TC × WR +

hree preparations, small bowel motor was determined by monitoring intra- luminal pressure with a fluid-filled, open-tipped polyethylene catheter connected externally to a pressure transducer. The lumen was filled with enough Tyrodes to maintain a pressure of 10–15 mm Hg. Lumen pressure was continuously monitored under control conditions and during 60- and 120-sec arterial occlusions.

Statistical analyses were performed by conventional methods, i.e., arithmetic mean ± SE, Student's *t* test.

Results. Under control conditions the systemic arterial pressure was 102.5 ± 6.9 mm Hg and the superior mesenteric blood flow was 28.6 ± 3.5 ml/min/100 g (as measured by the electromagnetic flowmeter) at a venous pressure of 5.0 mm Hg.

Figure 1 depicts the results of a representative experiment in which systemic arterial pressure (P_A), superior mesenteric arterial pressure (P_{MA}), venous pressure (P_V), and superior mesenteric blood flow (Q_B) were continuously monitored. When blood flow (31.0 ml/min/100 g) and the various perfusion pressures were in a steady state, ^{141}Ce

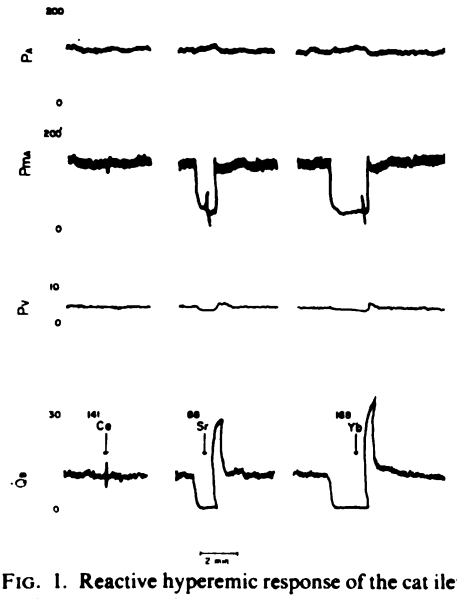

FIG. 1. Reactive hyperemic response of the cat ileum to graded arterial occlusions. Systemic arterial pressure (P_A), superior mesenteric arterial pressure (P_{MA}), venous outflow pressure (P_V), and total intestinal blood flow (Q_B) were continuously monitored. Radiolabeled microspheres were injected for determination of control (^{141}Ce), 60-sec (^{85}Cr), and 120-sec (^{169}Yb) arterial occlusion blood flow distributions.

microspheres were injected for control flow distribution within the various layers of the intestinal wall. Fifteen minutes later the arterial loop was occluded for 60 sec and ^{88}Sr microspheres were injected prior to releasing the occlusion. Upon release of the occlusion blood flow increased to a peak hyperemic level of 2.86 times control (86.1 ml/min/100 g). Fifteen minutes after all parameters returned to control levels the arterial loop was occluded for 120 sec (^{169}Yb microspheres were injected into the loop prior to releasing the occlusion). A peak hyperemic flow of 99.3 ml/min/100 g (3.3 × control) was observed for the 120-sec occlusion. For all experiments, occlusion of the superior mesenteric artery for 60 sec resulted in 2.24 ± 0.15-fold increase in total intestinal blood flow while 120-sec occlusions resulted in 3.06 ± 0.16-fold increase in flow. The duration of the reactive hyperemia for the 60- and 120-sec occlusions were 51.5 ± 2.1 and 76.2 ± 4.2 sec, respectively. Systemic arterial pressure was 109.8 ± 8.0 mm Hg during the arterial occlusions. In four separate experiments we observed no significant difference in the absolute blood flows and the relative blood flow distribution under steady-state conditions when the microspheres were administered in a random sequence. This suggests that the results obtained were due to the experimental interventions rather than differences among the microspheres.

The relative blood flow distribution in the mucosa–submucosal (M-S) and muscularis (M) layers of the intestine under control conditions was 64.5 ± 3.8% for M-S and 35.5 ± 3.8% for M. The cumulative results of the effect of 60- and 120-sec arterial occlusion on hyperemic flow to the mucosa–submucosa and muscularis layers are presented in Table I. The data indicates that 60-sec arterial occlusion causes a 2.10 ± 0.15-fold increase in M-S flow while M blood flow increases to 3.58 ± 0.83 times control. The difference between the M and M-S response to 60-sec arterial occlusion was not significant ($P > 0.10$). The 120-sec arterial occlusion causes a 2.48 ± 0.52-fold increase M-S blood flow while M blood flow increases to 6.30 ± 0.90 times control. Although the M-S response to 120-sec arterial occlusion was not significantly different than that observed after 60-sec occlusions, the M response to 120-sec occlusion was significantly greater than both the 120-sec M-S response ($P > 0.05$) and the 60-sec M response ($P > 0.01$). The results suggest that increasing the duration of the arterial occlusion does not significantly influence the response of the mucosa–submucosa vasculature yet has a significant effect on the muscularis circulation.

TABLE I. EFFECT OF ARTERIAL OCCLUSION ON THE REACTIVE HYPEREMIC RESPONSE IN THE MUCOSA–SUBMUCOSA AND MUSCULARIS LAYER OF THE CAT ILEUM

	Reactive hyperemia (× control)	
Occlusion time (sec)	Mucosa–submucosa[a]	Muscularis[b]
60	2.10 ± 0.15	3.58 ± 0.83
120	2.48 ± 0.52	6.30 ± 0.90*

[a] Absolute blood flow under control conditions = 22.8 ± 1.4 ml/min/100 g.

[b] Absolute blood flow under control conditions = 12.3 ± 1.4 ml/min/100 g.

* $P < 0.05$ When compared to the other layer of the same occlusion time, and when compared to the same layer and a different occlusion time.

An increase in motility and mean lumen pressure (tonus) was observed during 60- and 120-sec arterial occlusion when compared to the resting state. Although the mean lumen pressures achieved during 60- and 120-sec arterial occlusions were not significantly different; motility during 120-sec arterial occlusion (7 ± 1.2 contractions/min) was significantly greater than that observed during 60-sec occlusions (2.8 ± 0.6 contractions/min).

Discussion. The reactions of the intestinal microvasculature to vascular occlusion have been used to delineate the mechanisms involved in the regulation of intestinal blood flow, i.e., metabolic vs myogenic. An inherent assumption in previous studies was that the reactive hyperemic responses of the intestinal vasculature to graded arterial occlusion occur either uniformly in the mucosa, submucosa, and muscularis layers or predominantly in the mucosal layer. The magnitude of the reactive hyperemic response we observed at 60- and 120-sec arterial occlusions closely resemble previously reported values (2–4); however, the findings reported herein suggest that relatively uniform increases in intestinal wall blood flow occurs only for an occlusion period of 60 sec. Increasing the duration of

rterial occlusion to 120 sec results in a ɔminant hyperemia in the muscularis

e mechanism(s) by which increasing the ion of arterial occlusions increases the ive hyperemic response of the muscularis ılature is not clear but the increase in intestinal blood flow subsequent to re- of an arterial occlusion has been ex- ed by a combination of metabolic and genic mechanisms. It is difficult to ex- the results by a myogenic mechanism one would expect the reduced trans- l pressure at the microvascular level to ıe same, irrespective of the duration of ɔcclusion. An increased activity of vis- smooth muscle during longer occlusion ds would decrease vascular transmural ure and thus may account for the en- ed hyperemic response in the muscularis. ore likely explanation for the greater remia in the muscularis is a greater re- on in tissue oxygen tension in this layer 120-sec arterial occlusion. The results of tudies indicate that arterial occlusions of ıd 120 sec increase motility; however, a er increase in motility is observed during ec occlusions. Cessation of flow for 120 as also been shown to cause an increased ity in dog ileum (5). A greater oxygen it in the muscularis during the period of mia may be due to a greater demand for en by the tissue due to increased motility. ı and Grassmick (6) have recently dem- ated an increased percentage of total flow perfusing the muscularis-serosa ı motility is increased by manipulation, ntion, and physostigmine. Therefore, the er hyperemic response of the muscularis to 120-sec arterial occlusion may be due enhanced activity (motility) of visceral ıth muscle.

Irrespective of which mechanism(s) is responsible for the greater response of the muscularis layer to longer arterial occlusion periods, our results indicate that shorter occlusion periods are more suitable for interpretation of a reactive hyperemic response relative to metabolic and myogenic theories since a more uniform increase in intestinal wall blood flow occurs.

Summary. The reactive hyperemic response of the intestinal vasculature to graded arterial occlusion is often used to delineate mechanisms involved in the intrinsic regulation of intestinal blood flow. This study assesses if the reactive hyperemic response to 60- and 120-sec arterial occlusions occur uniformly throughout the wall of the small intestine. Radiolabeled microsphere injections were used to determine blood flow distribution in the mucosa–submucosa and muscularis layers of the small intestine under control conditions and during 60- and 120-sec arterial occlusion. The results of this study suggest that relatively uniform increases in intestinal wall blood flow occurs only for an occlusion period of 60 sec. Increasing the duration of the arterial occlusion to 120 sec results in a predominant hyperemia in the muscularis layer.

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Plasma Calcitonin in the Bovine Species (40635)[1]

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A variety of agents has been identified as calcitonin (CT) secretagogues during acute studies of their effects *in vivo* (1). However, few studies of the chronic regulation of calcitonin secretion have been reported. To pursue this, we have evaluated the effect of age, sex, and dietary calcium on plasma calcitonin in the bovine species. These studies demonstrate both similarities and differences of plasma calcitonin between the bovine species and other animal species.

Materials and methods. Blood samples were collected at random from the jugular vein from 169 (nonlactating) bovines of varying age, sex, and dietary calcium intake (Table I). Plasma was separated from each sample and stored frozen for subsequent measurements. Calcitonin was measured in each sample by a previously described radioimmunoassay procedure and calcium by atomic absorption spectrophotometry (2, 3). Student's *t* test was used for statistical analysis.

Results and discussion. Table I summarizes the results of the study. Calcium and calcitonin are represented as means (±SE) of at least triplicate determinations. These expanded studies confirm our earlier preliminary findings (2) that plasma CT is generally higher in bulls than in cows (Table I). The importance of gonadal factors in influencing plasma CT is further emphasized by the significantly ($P < 0.01$) lower calcitonin in steers (Table I, Group I) than in bulls and even in virgin females (Table I, Group H) of comparable age and dietary calcium intake who have similar plasma calcium concentrations. Sex-related differences in immunoassayable calcitonin have also been observed in other species. In the salmon and in the rat, plasma CT is higher in females (4–7), whereas in humans, males have higher plasma calcitonin (8, 9). In addition to sex, the current studies demonstrate that age also influences plasma CT: In the bovine species, younger animals tended to have higher plasma CT as well as calcium (Table I). In humans, higher levels of CT have also been reported in infants than adults and a decrease in calcitonin secretion as a function of age has recently been observed in adult females (10, 11). By contrast, in adult rats, plasma CT increases as a function of age (7, 12). These calcitonin differences may play some role in the differences of bone metabolism among animal species (13, 14).

Dietary calcium also seems to influence plasma CT in the bovine species. In general, bulls have higher plasma CT than cows whereas there were no consistent calcium differences between the sexes (Table I). More specifically, bulls of comparable age on a higher calcium diet (Table I, Group D) have significantly ($P < 0.01$) higher plasma CT than those on a lower calcium diet (Table I, Group B) despite no significant differences in calcium between the two groups. The high dietary calcium of the bovine species is generally designed for the increased needs of cows due to pregnancy and lactation. Thus, bulls ingest calcium which exceeds their physiological needs; in fact, it has been estimated that the dietary calcium need for a mature bull is 25 g/day (15). Because of this, it is reasonable to assume that the increased plasma CT in bulls represents a compensatory increase in hormone secretion to counter the excessive calcium ingestion.

The chronic hypercalcitoninemia in bulls may account for the progressive incidence of osteopetrosis which may involve most if not all of these animals past 7 years of age (16). The persistent C-cell stimulation may also

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TABLE I. PLASMA CALCITONIN AND CALCIUM IN THE BOVINE SPECIES

Group	Number	Animals	Age, years (mean ±SE)	Dietary calcium g/day	Calcitonin pg/ml (mean ±SE)	Calcium mg/dl (mean ±SE)
A[a]	20	Bulls	<6 (1.26 ± 0.05)	70	305 ± 12	10.29 ± 0.09
B	19	Bulls	≥6 (10.30 ± 0.49)	90	254 ± 10	9.70 ± 0.11
C	4	Bulls	<6 (3.38 ± 0.37)	110	294 ± 10	9.88 ± 0.09
D[b]	15	Bulls	≥6 (9.30 ± 0.48)	110	303 ± 12	9.59 ± 0.09
E[c]	14	Cows	<6 (3.33 ± 0.32)	200	186 ± 17	10.08 ± 0.06
F	10	Cows	≥6 (7.31 ± 0.65)	200	134 ± 8	9.71 ± 0.14
G	23	Virgins	<2 (1.03 ± 0.07)	78	264 ± 31	9.50 ± 0.06
H	30	Virgins	≥2 (3.4 ± 0.08)	56	240 ± 12	9.27 ± 0.07
I[d]	34	Steers	≥2 (3.3 ± 0.02)	56	190 ± 9	9.29 ± 0.07

[a] Significantly ($P < 0.01$) higher than B.
[b] Significantly ($P < 0.01$) higher than B.
[c] Significantly ($P < 0.05$) higher than F.
[d] Significantly ($P < 0.01$) lower than H and A–C.

ccount for the occurrence of medullary thyoid carcinoma in the bull (17). These views re supported by the fact that osteopetrosis nd medullary thyroid carcinoma occur exlusively in bulls and not in female bovines 16, 17). However, it is likely that other facts lay some role in at least the medullary thyoid carcinoma since this tumor can be acompanied by parathyroid hyperplasia and heochromocytomas, a syndrome remarkaly similar to the genetically transmitted mulple endocrine neoplasia, type II of humans 17).

These studies demonstrate that, as in other pecies, plasma CT in the bovine species is nfluenced by age and sex, and by diet. When ne results are compared to other animal udies, it is clear that species-specific differnces modulate the role of these factors in egulating plasma CT (1–10). Other factors nust also play a role in the sex differences of lasma calcitonin among different animal pecies.

Summary. Calcitonin (CT) was measured y radioimmunoassay in plasma samples rom 169 bovines of varying age, sex, and alcium diets. Younger animals had higher lasma CT than older animals. Bulls had igher plasma CT than cows or steers. Older ulls on a high calcium diet had higher lasma CT than those on a low calcium diet whereas there was no significant difference in lasma calcium between them. These studies lemonstrate that age, sex, and dietary calium can influence plasma CT in the bovine.

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Superoxide Dismutase Activity in the Crinkled Mutant Mouse: Ameliorative Effe Dietary Copper Supplementation (40636)

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Superoxide dismutase (S.O.D.) ($O_2^{\cdot -}$ - $O_2^{\cdot -}$ oxidoreductase EC 1.15.1.1.) catalyzes the disproportionation or dismutation of free $O_2^{\cdot -}$ radicals to yield hydrogen peroxide and oxygen (1, 2): ($O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$). The discovery of the activity of the enzyme was made by McCord and Fridovich (1) who showed that a copper- and zinc-containing protein, erythrocuprein, isolated from bovine red blood cells could catalyze the dismutation of superoxide ions. The protein was then assigned the more correct name S.O.D. This type of S.O.D. is generally found in the cytoplasm of all higher eukaryotic organisms (3) regardless of the cell type analyzed (4, 5). In addition to the cupro–zinc enzyme found in the cytoplasm, a manganese-containing enzyme having superoxide dismutase activity is found in the mitochondria of eukaryotic cells (3). It has been proposed that the physiological function of these enzymes is to protect oxygen-metabolizing organisms against the potentially deleterious effects of superoxide free radicals (6). The activity of cupro–zinc S.O.D. has been reported to be influenced by the level of dietary copper as inadequate levels of this trace element in the diet were associated with reduction of enzyme activity in swine (7) and in rats (8, 9).

The mouse homozygous for the autosomal recessive gene crinkled (*cr*) has several phenotypic characteristics similar to those seen in animals fed copper-deficient diets. The gene is characterized by decreased survival during the early postnatal period, alterations in skin thickness and pigmentation, decreased and retarded development of the hair bulbs, and neurological abnormalities (10, 11). Crinkled mice also show low liver copper concentrations from Day 18 of gestation to 21 days of age, but not thereafter (12). Diets high in copper (500 ppm) during pregnancy and lactation ameliorate many of the phenotypic traits of the gene (11–13).

In the present study, the activity copper metalloenzyme, S.O.D., was ured in livers of crinkled mice fed bc mal and high copper diets and compai the activity of the enzyme in nonc mice. Animals were examined at both 60 days of age, since liver copper cor tion was low before 21 days of age but thereafter (12). We also measured li pro–zinc S.O.D. activity in protein- ai deficient rat pups at 14 days of a control for morbidity, since both c groups also show high postnatal mort

Materials and methods. Known h gotic male and female carriers of the in a hybrid background were mate night. At the beginning of pregnancy, by the presence of vaginal plugs, were housed in individual cages ai domly assigned to one of three groups group, animals were given a commer leted stock diet (Purina Laboratory Ralston Purina Co., St. Louis, Mo.) c ing 12 ppm copper (by analysis). A group received a purified control di taining 6 ppm copper (by analysis), third group was fed the same purifi with the exception that it was supple with copper to a level of 500 ppm. Tl position of the complete purified c been reported previously (13). Dams v these diets throughout the experimei riod. Food and distilled water were p *ad libitum*. Temperature was mainta 68–72°F; lighting was on a 12-hr on–o

One crinkled (*cr/cr*) and one nonc (+/?) littermate control were killed fr litter at 14 days of age. Some litters female crinkled pups at 14 days of a for this reason, only males were test time of killing was restricted to 0800– in order to minimize possible diurna tions. The pups were anesthetized wit the thoracic cavity was exposed, the vena cava severed, and, in order to

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saline solution was perfused into the trium until the extremities appeared ivers were excised and weighed and a ıortion was kept for determination of : copper concentration while the re- ;r was homogenized in 0.32 *M* sucrose. ıove mitochondrial mangano-S.O.D., enates were centrifuged at 13,000 *g* for . The assay was then conducted on the atant.

ro–zinc superoxide dismutase activity termined by its ability to inhibit the idation of epinephrine to adreno- : at pH 10.2, according to the method ra and Fridovich (14). The reaction lowed spectrophotometrically using a l kinetic spectrophotometer at 480 nm. ıit of S.O.D. was taken as the concen- giving a 50% inhibition of the autoox- of epinephrine. The unit activity was ted from the linear portion of the plotting the reciprocal of the slope of ooxidation of epinephrine against the cal of homogenate added. Values are ed as activity/g tissue wet wt.

copper determination, samples of liver et ashed with 16 *N* nitric acid, concen- by evaporation, and diluted with ed distilled water. Diet samples were y ashed at 450°C for 24 hr and then as described above. Copper concen- was determined by atomic absorption photometry (Perkin–Elmer Model

nother experiment, the effect of age amined in mice fed the stock diet. At ; of age, one crinkled and one noncrin- ouse from each litter were killed and as described above.

est the effect of zinc or protein defi- on cupro–zinc S.O.D. activity, rats of ague–Dawley strain were used. Two of pregnant females received diets ıte in zinc (100 ppm) during gestation. turition, they were fed a purified diet ıing adequate zinc (100 ppm), or the urified diet deficient in zinc (less than n). The complete composition of the ıs reported previously (15). At 14 days rat pups from each group were killed er S.O.D. activity was determined as ed above. In addition, plasma zinc was ed by atomic absorption spectropho- tometry after dilution with distilled water.

Additional rats were fed during gestation purified diets which were either adequate in protein (24%), or were protein deficient (4%). After parturition, both groups were fed the diet adequate in protein (24%). Fourteen days postnatally, pups were taken from both groups and liver cupro–zinc S.O.D. activity was determined as previously described. The complete composition of the diet has been published (16).

Statistical analyses. The significance of the difference between arithmetic means was evaluated by Student's *t* test.

Results. In the litters of dams fed either the stock diet or the purified control diet, liver cupro–zinc S.O.D. activity was significantly lower in the crinkled mutants than in their littermate controls, by about 30%. However, in crinkled mutants from dams receiving the copper supplemented diet (500 ppm Cu), activity of the enzyme was normal, the same as their littermate controls (Table I). Furthermore, copper supplementation did not produce an increased activity of the enzyme in noncrinkled mice over the values found in mice from stock fed or purified control diet fed dams (Table I).

In the litters of dams fed either the stock diet or the purified control diet, liver copper concentration was significantly lower at 14 days of age in crinkled mutants than in their littermate controls (Table I). There was no significant difference in liver copper levels between crinkled and noncrinkled mice from dams fed the copper-supplemented diet (Table I). As with cupro–zinc S.O.D. activity, there was no significant difference in liver copper concentrations of noncrinkled mice in any of the dietary groups (Table I). At 60 days of age, crinkled mutants showed significantly lower liver cupro–zinc S.O.D. activity than their littermate controls while liver copper levels were similar (Table I).

Cupro–zinc superoxide dismutase activity was not found to be significantly different between zinc-sufficient and zinc-deficient rat pups, although the plasma zinc concentrations indicated a severe deficiency (Table II). Similarly, cupro–zinc S.O.D. activity was not depressed by prenatal protein deficiency (Table II).

Discussion. It is apparent that one of the

TABLE I. SUPEROXIDE DISMUTASE ACTIVITY AND COPPER CONCENTRATION IN LIVER OF CRINKLED (cr/cr) AND NONCRINKLED (+/?) MICE

Diet	Experimental group	N	Superoxide dismutase (units/g wet wt)	Copper (μg/g wet wt)
Stock (12 ppm Cu)	14 days old			
	+/?	4	691 ± 109[a]	22.8 ± 1.2[a]
	cr/cr	4	388 ± 59[b]	17.5 ± 1.8[b]
Purified control (6 ppm Cu)	14 days old			
	+/?	4	590 ± 69[a]	25.4 ± 2.4[a]
	cr/cr	4	406 ± 41[b]	13.4 ± 2.1[b]
	60 days old			
	+/?	5	580 ± 40[a]	4.2 ± 0.5[c, d]
	cr/cr	5	420 ± 32[b]	4.4 ± 0.4[c, d]
Copper supplemented (500 ppm Cu)	14 days old			
	+/?	4	581 ± 77[a]	23.4 ± 2.0[a]
	cr/cr	4	650 ± 61[a]	28.3 ± 4.0[a]

[a, b, c] Values are expressed as mean ± SE of the mean. Values with different superscripts are significantly different, $P < 0.05$ (Student's t test).
[d] Data from three animals.

TABLE II. LIVER SUPEROXIDE DISMUTASE ACTIVITY AND PLASMA ZINC CONCENTRATIONS IN 14-DAY-OLD RATS

Diet	N	Superoxide dismutase (units/g wet wt)	Plasma (μg zinc/dl)[a]
100 ppm Zn	6	464 ± 34	116 ± 7
0 ppm Zn	6	448 ± 24	60 ± 5[b]
24% Protein	4	487 ± 33	—
4% Protein	4	488 ± 29	—

[a] Values are expressed as mean ± SE of the mean.
[b] Significantly lower than control at $P < 0.01$ (Student's t test).

phenotypic expressions of the gene *cr* is a lower than normal activity of the enzyme cupro–zinc S.O.D. To determine if the low activity of the enzyme in the crinkled mutant is the result of a mutation in the S.O.D. gene itself, we have examined the electrostatic properties of the cupro–zinc S.O.D. isoenzymes by isoelectric focusing. There was no difference found in the isoenzyme pattern between the crinkled and noncrinkled mouse (unpublished results), which suggests that the low activity of the enzyme in the crinkled animals is due to the mutants poor copper status, and not to a specific S.O.D. gene mutation. While there have been reports dealing with abnormal isoenzyme variants of S.O.D. in humans, it is not clear that the activity of the enzyme is altered in these individuals (17–19). We believe our report is the first to demonstrate a depression of S.O.D. activity in a genetic mutant and its increase to normal levels by means of dietary manipulation.

The two identified errors of copper metabolism in the crinkled mouse, low liver copper concentration and abnormal copper intestinal transport, are not detectable past the early postnatal period (12, 20). Since copper concentration in the liver was normal after 21 days of age, it was surprising that the low activity of cupro–zinc S.O.D. in the nonsupplemented mutant persisted beyond this time. This suggests that cupro–zinc S.O.D. activity may be permanently affected by the early nutritional environment of the animal. Similar sustained pertubations have been reported to occur for the enzymes 3-hydroxy-3-methylglutaryl coenzyme A reductase (21), acetyl-CoA carboxylase (22), alkaline phosphatase, and disaccharidase (23), in rats exposed to varying nutritional environments.

The finding of low liver copper concentrations in the nonsupplemented mutants, while liver copper concentration and cupro–zinc S.O.D. activity in the copper supplemented mutants were similar to littermate controls, supports the idea that low activity of the enzyme in the mutant is correlated with an abnormality in copper metabolism. The finding of normal levels of cupro–zinc S.O.D. activity in both zinc-deficient and protein-deficient rat pups argues against the hypothesis that low levels of cupro–zinc S.O.D. activity are a common sign in states of nutritional inadequacy. The important finding

decreased copper levels in the mutant
e correlated with decreased cupro–zinc
). activity emphasizes the need to per-
studies on other copper-dependent en-
s in this mutant. Since it has been pro-
l that low levels of S.O.D. activity can
leterious to oxygen-metabolizing orga-
s (6), the combined effect of this insult
her with decreased activities of other
r-dependent enzymes could explain the
viability of the mutant.

ce the report of McCord and Fridovich
ggesting that the enzymatic function of
olue copper protein "erythrocuprein"
be to catalyze the dismutation of su-
ide free radical anions, interest in the
ne has been steadily mounting. While
nzyme is generally believed to have a
ctive function, its precise biological role
defined (24). One method of attempting
fine better the role(s) of S.O.D. is to
it in normal or pathological situations
its activity is altered or in a state of
For example, an increase in S.O.D. ac-
has been found to precede rapid cell
th in *Escherichia coli* (25), while a de-
e in S.O.D. activity with age has been
sted as a causative factor for age-related
ge to cells in mice (26). Bonta has re-
d that S.O.D. activity in human cord
l is inversely related to gestational age,
a rapid increase in activity occurs
ly after birth, presumably triggered by
xposure of the neonate to high oxygen
ons. Failure of this process to occur has
suggested as part of the etiology of
t respiratory distress syndrome (27).
uations in S.O.D. activity have also been
lated recently with chronic infection
ias in the dog (28), the occurrence of
natoid arthritis (29), diabetes (30), and
itutional infantile panmyelopathy in
(31).

s obvious that a more complete under-
ing of the activity and control of S.O.D.
essary, not only with regard to its role
rmal development, metabolism, and ag-
ut also because of its putative involve-
in several pathological conditions. The
g that cupro–zinc S.O.D. activity is de-
ed in the crinkled mutant, and that it
e increased to normal levels by dietary
r supplementation may allow this mu-
tant to serve as a tool in studies on control mechanisms for the enzyme, as well as on the effects of its deficiency.

Summary. Liver cupro–zinc superoxide dismutase activity was lower in mice homozygous for the gene *crinkled* (*cr*) than in noncrinkled littermate controls (+/?), both at 14 and at 60 days of age. Liver copper concentration was also low at 14 days, but was normal at 60 days. Supplementation during pregnancy and lactation with a high level of dietary copper brought both cupro–zinc S.O.D. activity and copper concentration in the liver of mutant offspring to levels similar to those of nonmutant littermate controls. Liver cupro–zinc superoxide dismutase activity was not found to be influenced by a deficiency of either zinc or protein in the rat.

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tric Inhibitory Polypeptide, Insulin, and Glucose Changes Produced by Growth Hormone, Prednisolone, Glucagon, Insulin, Fasting, or Diazoxide (40637)[1]

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: gastric inhibitory polypeptide (GIP) nts glucose-stimulated insulin secre--5), and since the amount of plasma is important in diabetes, hypoglyce-nd other clinical disorders, we have gated effects on plasma GIP levels of s of compounds and metabolic states ve been found to affect insulin release. ited the effects of growth hormone, eroid (prednisolone), and glucagon; cts of epinephrine are presented in a e report (unpublished). Each of these nes can influence the secretion and net of insulin and are involved signifi-in preventing hypoglycemia, whether ted with a high absolute level of insu-ction, insulinoma) or relative increase We included studies of prolonged fast-d of diazoxide; the latter was studied e of its inhibitory action on insulin on (6). We sought correlations of s in plasma GIP, insulin, and glucose.

erials and methods. Dogs. Five or more / purebred female foxhounds weighing n 17 and 21 kg were used for each nent. Three days or more elapsed be-tests. After a 16-hr fast, they were ined in a calm and relaxed state in jackets. A 20-gauge plastic cannula serted into the cephalic vein and was atent by a slow drip of normal saline. each test compound was given, two or baseline blood specimens were ob-5 min apart. Growth hormone, glu-insulin, or diazoxide was infused at a nt rate (Harvard pump) into the ce-phalic vein of the opposite leg. Glucose and prednisolone were given by gastric tube (prednisolone also was injected intramuscularly). All compounds given orally were in a total volume of 200 ml of warm water. In control experiments, this amount of water alone was used. Each oral dose of glucose was 1 g/kg. The dosages of the other test substances and additional details of the experiments are presented in the legends with the figures.

Blood aliquots for GIP, insulin, and glucose assays were placed in EDTA; those for glucagon were put in tubes with heparin and 0.05 ml benzamidine (1 *M*). All tubes were submerged immediately in ice. Plasma for glucagon was separated within 1 hr and for the other tests within 2 or 3 hr. All specimens were stored at −70° until assayed.

Supplies. The antibody for glucagon (30 K) was kindly supplied by Dr. Roger Unger (Veterans Hospital, Dallas) and that for GIP by Drs. John Brown (University of British Columbia) and Werner Creutzfeldt (University of Göttingen). GIP was obtained from Dr. John Brown, growth hormone (bovine) from the National Institutes of Health, insulin and glucagon from the Eli Lilly Company, benzamidine from the Aldrich Chemical Company, and diazoxide from the Schering Corporation.

Assays. Glucose was assayed by the glucose oxidase method, using the Beckman Analyzer. GIP, insulin, and glucagon were measured by radioimmunoassay, using the respective methods of Kuzio *et al.* (8), Morgan and Lazarow (9), and Ensinck *et al.* (10). With the antibodies used there was no significant cross-reactivity by secretin, cholecystokinin, neurotensin, substance P, vasoactive intestinal polypeptide, bombesin, growth hormone, or gastrin. Insulin, glucagon, and GIP reacted significantly only with its respective antibody. All assays were run in duplicate. The lower limit of sensitivity for the GIP

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1 technical assistance of Jan Biesbroeck and hampagne.

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assay was 125 pg/ml. All of the specimens assayed had values significantly higher than this. Intraassay variation was 9% and interassay variation was 20%.

Presentation of data. In the figures are presented the mean absolute values (±SEM) for glucose, but for GIP the mean of the fasting values for each dog is calculated and the mean difference (±SEM) from this zero point is plotted for each time point. The data for insulin and glucagon are presented likewise. This was done to reconcile the interanimal variations in baseline levels of hormones. Typical mean baseline levels (±SEM) were: GIP, 455 ± 41 pg/ml; insulin, 7 ± 1 μU/ml; and glucagon, 40 ± 5 pg/ml. The statistical significance of the responses was determined by a paired Student's *t* test on the natural logarithm between baseline and response levels in each dog. Logarithmic transformation was performed to approximate better the Gaussian distribution required for this parametric statistical analysis. As needed, an asterisk or other symbol is used in the figures to indicate significant differences ($P < 0.05$). In the text all reports of observed changes are statistically significant to this extent, or more.

Results. Effect of growth hormone or prednisolone. Growth hormone alone increased plasma insulin and slightly decreased GIP, but produced no change in glucose (Fig. 1). It decreased the early increments in plasma GIP and glucose produced by glucose ($P < 0.05$), but did not affect significantly the increase in insulin.

Prednisolone alone did not change the levels of GIP, insulin, or glucose, and did not affect glucose-induced increments in plasma GIP or insulin (Fig. 1), but it slightly decreased the early rise in plasma glucose.

Effect of glucagon. Infusion of glucagon (5 ng/kg/min) for 15 min increased plasma insulin and markedly increased plasma glucagon, but did not change GIP or glucose levels (Fig. 2). Continuation of this infusion for 60 min after oral glucose did not affect significantly glucose-induced rises in plasma GIP, insulin, or glucose. Infusion of a large dose of glucagon, 50 ng/kg/min for 60 min, beginning 15 min after oral glucose, caused no significant inhibition of glucose-stimulated rises in plasma GIP. There were increments in insulin >400 μU/ml. and in glucose >100

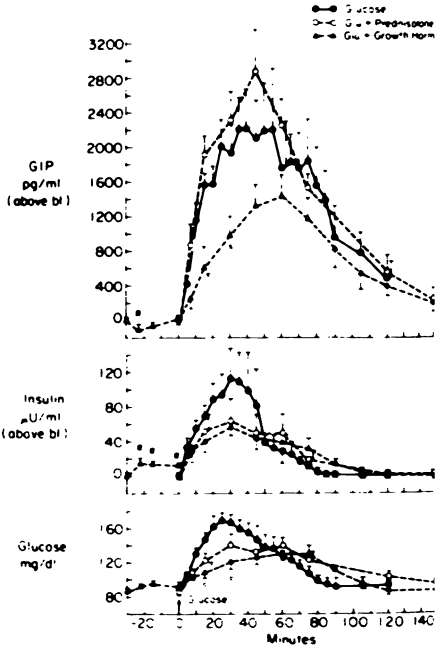

FIG. 1. There were three series of experiments, of which each dog was given glucose, 1 g/kg, by tube at time 0. In one series, growth hormone (0.(kg/min) was infused for 1 hr, and glucose was gi min after starting the infusion. Blood specimen acquired periodically during a period of 3 hr. A series of experiments tested the effects of predni alone, followed by oral glucose. Fasting blood spe were obtained, at 8:00 and 8:05 AM, on the day pre the main test. At 4:00 and 11:00 PM on that day 6:00 AM on the following day prednisolone sodium phate, 20 mg, was injected intramuscularly. Ther blood was drawn at 8:00 and 8:05 AM. Then gluco prednisolone, 20 mg, were given by gastric tub third series of experiments dogs were given only g

The mean and SEM for each time are plotted. ues that differed ($P < 0.05$) from baseline values ob before any infusion of growth hormone. * Deviat < 0.05) from results found with oral glucose alon pretreatment baseline values (for times −5 and 0 averaged and shown as one point. The baseline obtained before prednisolone treatment are not they differed little from the results obtained fron of specimens drawn immediately before oral gluc

mg/dl.

Effect of insulin. Although intravenou fusion of insulin, 57 mU/kg/min for 35 increased plasma IRI to levels >300 μ and reduced plasma glucose to levels mg/dl, it did not change plasma GIP significantly (Fig. 3). Moreover, infusi insulin, 30 mU/kg/min, starting 20 mi

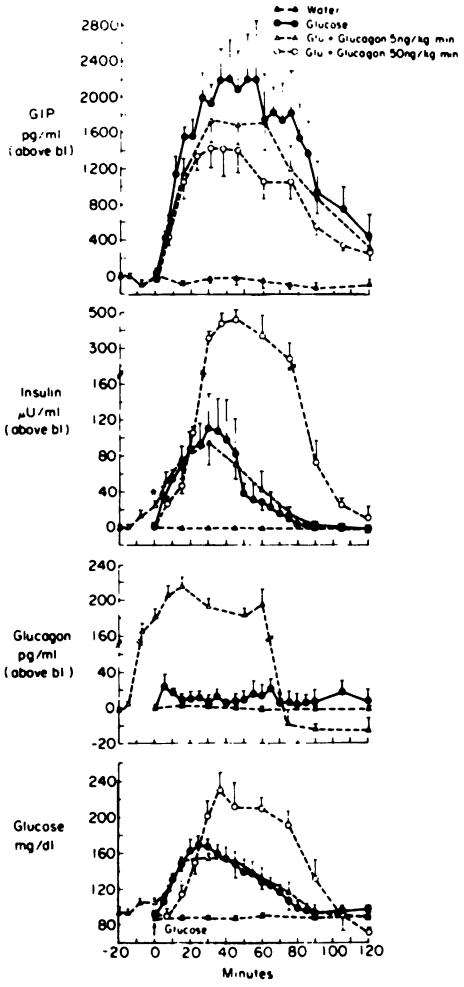

FIG. 2. Results from four series of experiments are shown. At time 0 one group was given only water by gastric tube. Three other series received either: (a) glucose alone, (b) glucose plus glucagon, 5 ng/kg/min (infused from −15 to 60 min), or (c) glucose plus glucagon, 50 ng/kg/min (infused from 15 to 75 min). All glucose (1 g/kg) was given by gastric tube. * Deviation from baseline values ($P < 0.05$).

fore oral glucose and continuing for 45 min after the glucose, did not alter the basal levels of GIP nor the GIP rises produced by glucose, despite increases in plasma insulin >100 μU/ml.

Effect of prolonged fasting or diazoxide. Fasting for 4 days plus the routine 16-hr fast did not change the plasma insulin or glucose levels, but decreased GIP levels ($P < 0.05$) (Fig. 4). Whereas glucose caused as much increase in GIP concentrations in dogs with the long fast as with the short fast, the actual levels attained were not as high during the early periods. After the long fast the mean basal glucagon level was 45.9 ± 4.2 pg/ml, which was comparable to that for the 16-hr fast (not plotted). Oral glucose given to long-fasted dogs caused a more prolonged rise in plasma glucose and insulin than it produced in dogs fasted for 16 hr. Diazoxide increased fasting plasma glucose (Fig. 4) but did not change plasma insulin or GIP. Compared with glucose alone, diazoxide plus glucose caused less insulin secretion, a slower rise in plasma GIP, and a more prolonged increase in glucose (Fig. 4). Diazoxide produced no significant changes in plasma glucagon levels

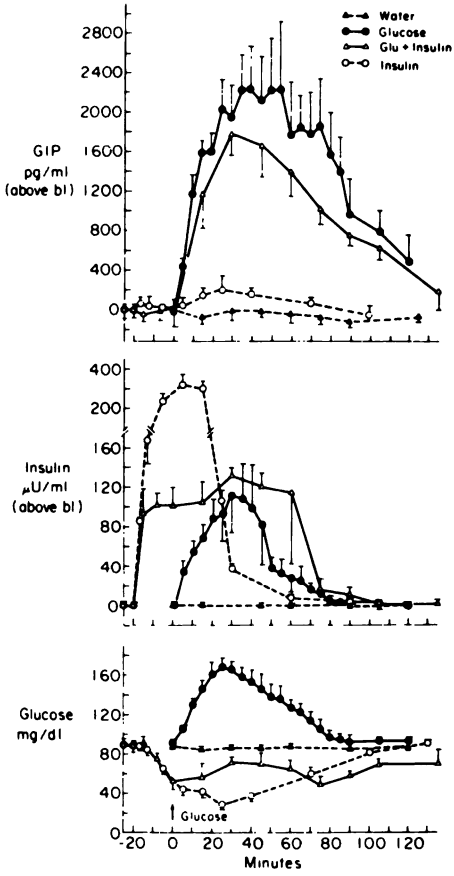

FIG. 3. Results from four series of experiments are shown. At time 0: one series was given water alone; another series was given glucose alone; a third series received glucose plus insulin, 30 mU/kg/min from −20 to 45 min. The fourth series received insulin alone, 57 mU/kg/min, from −20 to 15 min. The water and glucose, 1 g/kg, were given by gastric tube.

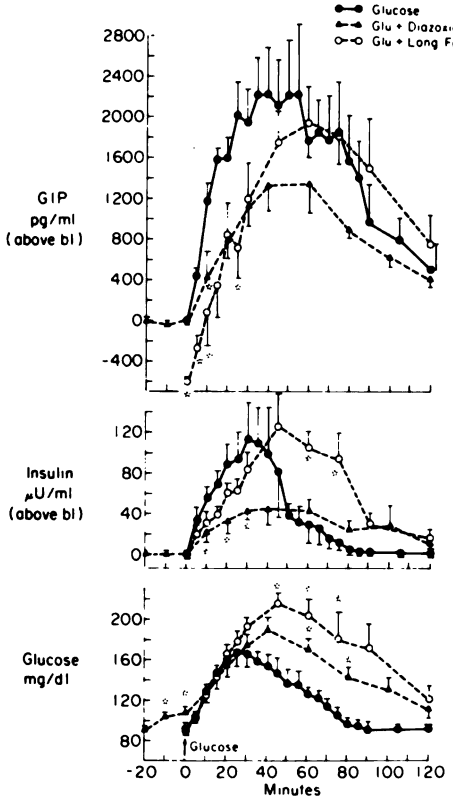

FIG. 4. In each of three series of experiments oral glucose, 1 g/kg, was given by gastric tube at time 0. In two series, glucose alone was given to dogs fasted for 16 hr or for 112 hr. The third series, fasted for 16 hr, were infused with diazoxide (5 mg/kg within 2 min), beginning 20 min before oral glucose. The mean decrease in GIP between a 16- and 112-hr fast was 600 pg/ml, and the baseline for the latter is shown at this level. * Differences ($P < 0.05$) in results obtained when glucose alone was given to dogs fasted 16 hr and those given glucose after a long fast or after diazoxide.

before or after glucose administration (not plotted).

Discussion. As indicated earlier, insulin secretion can be influenced by GIP and each of the factors that we studied (GH, prednisolone, glucagon, fasting, and diazoxide). In our studies we were interested most in correlating changes in plasma GIP, insulin, and glucose, observing especially how many of the factors influenced plasma GIP levels and evaluating the role of GIP in producing changes in insulin. Since the effects of GIP on plasma glucose result from its stimulation of the secretion of insulin and glucagon (3, 11), and these hormones have been considered to decrease GIP secretion (3, 12), we investigated these effects by infusing insulin and glucagon.

We found that infusion of GH alone increased plasma insulin yet decreased GIP. Since GH infusion decreased the early increase of plasma GIP, insulin, and glucose produced by oral glucose, we ask whether these changes were attributable to: (a) an increase in gastrointestinal transit time and/or decrease in intestinal absorption of glucose, (b) increased glucose utilization by peripheral tissues, (c) rapid increase in insulin release resulting from GH action on B cells, or (d) inhibition of GIP secretion by insulin, glucagon, or GH? Since GH promotes rapid release of insulin and glucagon from perfused pancreas (13), the GH-stimulated early rise in insulin presumably resulted from GH action on islet cells and was not mediated by GIP. GH can cause increased glucose uptake by muscle and adipose tissue by its action on these tissues (10). This action plus the early insulin increase may have accounted for the decreased early rise in plasma glucose; GH is known to decrease plasma glucose even without increase in plasma insulin (14). In view of the failure of large doses of insulin (Fig. 3) and glucagon (Fig. 2) to inhibit glucose-induced GIP rises, the slow increase in GIP that occurred with GH plus glucose does not seem to be related to effects of insulin or glucagon. Possibly GH caused some delay in gastrointestinal absorption of oral glucose. In two experiments in which we included [^{14}C]glucose with the oral glucose and followed ^{14}C levels in the plasma, the apogee was about 15 min later than with glucose alone (five tests); this possibly could be due partially to delayed absorption and/or GH-stimulated increase in glucose utilization.

Prednisolone did not affect baseline levels of GIP, insulin, or glucose, nor glucose-induced rises in GIP and insulin. However, it slightly decreased the early rise in plasma glucose. The basis for this is unknown; others have found that glucosteroid given for 3 days exaggerated plasma glucose and insulin rises after glucose administration (15).

Glucagon, 5 mg/kg/min, did not affect fasting GIP levels or its response to oral glucose. Moreover, when glucagon was infused in a pharmacologic dose, 50 mg/kg/min, starting 15 min after oral glucose, it did

it significantly the GIP rise. Ebert *et* found that this large dose did not sting GIP levels in man, but that infusion was begun before a large eal it inhibited GIP increase. How- hey signified, this large dose is un- gical and could inhibit gastric emp- en our smaller dose, 5 mg/kg/min, l increments in plasma glucagon /ml, which are higher than we have with physiologic amounts of GIP. e, it is questioned whether glucagon exerts a significant role in control- secretion.

d diabetics we have found normal onses to glucose (13), but moderately iabetics had hypernormal GIP re- (3, 17, 18). Since insulin treatment the responses (3), it has been sug- at insulin exerts feedback inhibition secretion. However, this inhibition indirect, because diabetes has been increase glucose absorption from ine (19), and possibly in this manner GIP release.

ett *et al.* (20) found that insulin in- glucose-stimulated GIP rise, while *t al.* (21) did not observe this to occur poglycemia developed. However, we decrease in plasma GIP levels pro- y insulin, regardless of whether in- s given alone or with glucose, or hypoglycemia or euglycemia was As discussed earlier, hypoglycemia ase the secretion of GH, glucagon, oid, and epinephrine. In testing rmones, we found that only GH af- e GIP response to glucose.

tide decreased glucose-stimulated ecretion and increased plasma glu- e increase in glucose results from d insulin secretion, increased glucose on, and inhibition of glucose uptake ether the inhibition of GIP is a direct ct action of diazoxide is unknown. n of pancreas with diazoxide de- he secretion of glucagon, somatosta- reatic polypeptide, and insulin (19). und that fasting for 112 hr signifi- ecreased plasma GIP, but did not nsulin or glucose. Whereas this fast l the duration of glucose-induced lasma insulin and glucose, it did not change the amount of GIP rise. Therefore, the persistence of the hyperinsulinemia did not inhibit the increase in GIP. Wilms *et al.* (24) found that fasting obese patients caused plasma GIP levels to increase and insulin to decrease within a few days. Despite the rise in baseline GIP, these fasted subjects experienced a decrease in glucose-induced GIP response. To what extent obesity and other factors accounted for the difference in results in obese patients and those in our nonobese dogs is unclear.

In conclusion, whereas GIP can stimulate insulin secretion, there are many factors that increase plasma insulin without increasing plasma GIP. Moreover, increase in plasma insulin may not suppress glucose-induced increase in plasma GIP.

Summary. Because of the strong augmentary action of gastric inhibitory polypeptide (GIP) on insulin secretion, we studied in foxhounds the effect on GIP secretion of several factors known to affect insulin secretion and plasma glucose. Growth hormone infusion decreased plasma GIP, yet increased insulin; no change in plasma glucose was found. Growth hormone inhibited oral glucose-induced increments in plasma GIP and glucose. Prednisolone did not affect levels of GIP or insulin, whether given alone or preceding oral glucose. Diazoxide decreased glucose-induced GIP and insulin secretion, but increased plasma glucose. Fasting for 112 hr decreased GIP but did not decrease the glucose-induced GIP rise. Infusion of glucagon or insulin in amounts sufficient to produce high physiologic or pharmacologic plasma levels did not change GIP concentration, nor did they alter GIP rises with glucose. In conclusion, GIP was not inhibited by insulin or glucagon but was decreased by growth hormone, fasting, or diazoxide. Whereas fasting increased glucose-stimulated insulin secretion, diazoxide decreased it. The insulin-releasing activity of a number of factors does not correlate with increases in plasma GIP, and increases in plasma insulin or glucagon may not suppress increases in plasma GIP. There was not a good correlation of the plasma levels of GIP, insulin, glucose, and glucagon.

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ɔne Fluoride: Acute Hepatotoxicity in Rats Pretreated with PCB or Phenobarbital (40638)[1]

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idene fluoride (VDF: 1,1-difluoro-ɛ) is an important plastics monomer. produced by dehydrochlorination of ifluoroethane at 600°F. Its polymeri-ɔroducts include poly-VDF and the ers fluorinated ethylene–propylene lychlorotrifluorethylene. In 1970 the ɔduction of fluorocarbon resins in the ɛcluding polytetrafluoroethylene, was × 10^6 lbs. (1). Occupational exposure is not regulated by a specific thresh-ɪt value (2), reflecting the fact that it ɛgarded as a compound of significant

reports have been published on the ɔgy of VDF. Carpenter *et al.* (3) found ⊦hr inhalation of 128,000 ppm VDF ɪtely lethal to rats. However, Lester ɛenberg (4) observed only an unsteady rats exposed to 80% (800,000 ppm) 20% O_2 for 19 hr. The postural reflex ɪsciousness were not affected. Jaeger) confirmed this lack of obvious acute by exposing rats to 82,000 ppm VDF r. No mutagenicity of VDF was de-n bacteria by Bartsch *et al.* (6). They led to find any evidence that VDF is lically activated to alkylating inter-ɛs using a 4-nitro(4-benzyl)pyridine g system. Evidence that VDF is me-ɛd *in vivo* and that it can affect renal ɪ in rats was published by Dilley *et al.* ɛy exposed rats to 2200 ppm VDF for and found significant increases in excretion of fluoride and potassium ions. Urinary protein levels were also increased after this exposure.

Ethylene, vinyl fluoride, vinyl chloride, and vinyl bromide are plastics monomers that share with VDF both structural similarities and a lack of acute toxicity. We have previously shown, however, that ethylene and its monohalogenated analogs are acutely hepatotoxic in rats pretreated with polychlorinated biphenyl (PCB) (8). PCB is a potent inducer of the hepatic mixed-function oxidase system (MFOS) (9). The similarities between VDF and these hepatotoxic monomers, as well as their lack of acute toxicity in normal (not pretreated) rats, led us to investigate the influence of the MFOS inducers PCB and phenobarbital (PB) on VDF hepatotoxicity. This toxicity was evaluated by measurement of liver weight, serum sorbital dehydrogenase (SDH) activity, and by light microscopic examination of the liver. SDH is a sensitive and specific index of acute hepatic injury (11).

Methods. Animals. Male Holtzman rats, 170–250 g, housed five or six per cage, were maintained on a 12-hr light–dark cycle. They were supplied with commercial rat chow (Purina Rat Chow, Ralston Purina Co., St. Louis, Mo.) and tap water *ad libitum.* The chow was removed 18 hr before VDF exposure. During exposure neither water nor chow were available. After exposure only water was allowed. Rats were sacrificed by cervical transection 24 hr after VDF exposure. Experience with compounds of similar structure and toxicity had suggested that the 24 hr interval was sufficient for injury to develop (10).

PCB and phenobarbital treatments. PCB (Aroclor 1254, Monsanto Chemical Co., St. Louis, Mo.) was solubilized in a vehicle of water containing 0.5% (w/v) methyl cellulose and 0.5% (v/v) Tween 80. Between 2:00 and 5:00 PM each day the rats were given 100 mg

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PCB/kg by gavage for 3 consecutive days. VDF exposure was on the day following the last PCB treatment. PB (0.1% w/v) was supplied in the drinking water for the 7 days prior to VDF exposure.

VDF exposure. VDF (99% minimum purity, Matheson Gas Company, Gloucester, Mass.) inhalation began between 9:00 and 11:00 AM and lasted 4 or 6 hr. The inhalation chambers and analytical methodology were as previously described (8).

Sorbitol dehydrogenase assay. Sorbitol dehydrogenase activity was measured with a method modified from that of Gerlach and Hiby (11). Serum (0.3 ml or an appropriate dilution thereof), triethanolamine buffer (2.3 ml, 0.3 *M*, pH 7.4), and NADH (0.1 ml, 12 m*M* in 0.1% NaHCO3) were mixed together and incubated at 30°C for 30 min. At the end of this incubation D(+)−fructose (0.3 ml., 4.0 *M*) was added. The disappearance of NADH absorbance was monitored at 366 nm by a Gilford 240 spectrophotometer equipped with a Gilford 410 digital absorbance meter.

Histological preparation. Slices of liver were fixed by immersion in 10% aqueous buffered formaldehyde. Paraffin-embedded sections (5–6 μm) were stained with hematoxylin and eosin.

Data presentation and analysis. Liver weight data are presented as g liver/100 g body wt to correct for differences in rat weights. Statistical evaluation of liver weight and SDH values are based on the logarithmic transformation of the raw data to assure similarity of variances. The data are presented as the geometric mean and standard error range. Differences between means were evaluated using the independent *t* test (12). A *P* value of less than 0.05 was considered to be significant.

Results. Gross inspection of rats exposed to VDF revealed hepatotoxic effects only in animals pretreated with PCB. In severe injury, intermingled areas of pallor and congestion were seen in a brittle liver. Light microscopic examination showed VDF-related hepatic damage (centrolobular to midzonal ballooning and necrosis) which was severe in rats pretreated with PCB and relatively mild in PB-pretreated rats. The ballooning of hepatocytes probably represents severe degeneration, i.e., endstage changes leading to cell death or necrosis. No gross or microscopic effects of VDF on other organs were seen regardless of pretreatment.

Hepatotoxicity and mortality after VDF exposure. No pretreatment: No effect was detectable on liver weight, SDH, and mortality in rats exposed to 25,000 ppm VDF for 6 hr with no preexposure treatment (Table I).

Phenobarbital pretreatment. Table I shows that 25,000 ppm VDF for 6 hr significantly increased liver weight in PB-pretreated rats. SDH and mortality were not affected.

Polychlorinated biphenyl pretreatment. Four-hour VDF exposure of rats pretreated with PCB caused increased liver weight at all of the concentrations tested. This effect was statistically significant at 15,000 and 25,000

TABLE I. ACUTE HEPATOTOXICITY OF VINYLIDENE FLUORIDE IN FASTED RATS PRETREATED WITH POLYCHLORINATED BIPHENYL[a] OR WITH PHENOBARBITAL[b]

Pretreatment	Vinylidene fluoride exposure (ppm)	Duration of exposure (hr)	Liver weight (g/100 g body wt)	SDH	Mortality
None	none	—	3.25 (3.16–3.33)	23.9 (19.9–28.7)	0/5
None	25,000	6	3.38 (3.34–3.43)	9.3 (8.7–9.8)	0/6
PB	None	—	4.27 (4.19–4.36)	20.6 (19.3–22.0)	0/6
PB	25,000	6	4.88 (4.76–5.01)[c]	14.6 (13.1–16.2)	0/5
Tween 80-methyl cellulose vehicle	None	—	3.21 (3.20–3.22)	11.7 (11.4–12.3)	0/4
PCB	None	—	6.59 (6.44–6.75)	16.1 (14.9–17.4)	0/6
PCB	5,000	4	7.70 (7.23–8.20)	63.6 (49.9–81.1)[d]	0/9
PCB	15,000	4	9.91 (9.54–10.29)[d]	137 (114–163)[d]	0/9
PCB	25,000	4	9.57 (9.05–10.12)[d]	281 (208–381)[d]	2/6

[a] Polychlorinated biphenyl (PCB), 100 mg/kg once a day for 3 consecutive days by gavage.
[b] Phenobarbital (PB), 0.1% (w/v) in drinking water for 7 days prior to exposure.
[c] Significantly different ($P < 0.01$ by the independent *t* test) from PB treated, not exposed.
[d] Significantly different ($P < 0.01$ by the independent *t* test) from PCB treated, not exposed.

not at 5000 ppm. The increase in ght was dose dependent from 5000 ppm VDF, but not from 15,000 to DF. SDH values were elevated sig- and in a dose-dependent fashion at DF concentrations tested. No signs is nor of other gross abnormalities ed at the end of VDF exposure. in the group of PCB-pretreated rats o 25,000 ppm VDF, two out of six found dead the following morning, after the beginning of VDF expo- le I).

gic changes. Phenobarbital treated, ed. Phenobarbital did not alter the oscopic appearance of the liver.

eated, not exposed. The methyl cel- veen 80 vehicle itself had no effect stology of the liver. PCB treatment toplasmic vacuolization of centro- nd midzonal areas (Fig. 6) (8). In there were a few foci of subcapsular e ballooning and necrosis. The bal- s had pyknotic nuclei peripherally by an unstained vacuole which al- pletely replaced the normal cyto- ecrotic areas were interspersed with ebris and a few polymorphonuclear s.

25,000 ppm), not pretreated. No ap- effect was noted on the histologic ce of liver in rats which received m VDF for 6 hr without pretreat- gs. 1 and 5).

arbital + VDF (25,000 ppm). In sub- areas hepatocytes with pale cyto- owed grouping of chromatin along ar membrane. In other peripheral allooning liver cells surrounded rare flammatory reaction with necrosis The balloon cells were identical in ce to those occasionally seen in the ated only with PCB. Mild conges- seen in the deep hepatic paren-

VDF (5000 ppm). The PCB-related tion of hepatocytes was accompa- extensive centrolobular ballooning olving half of the lobules (Fig. 7). ty-looking hepatocytes had scanty n with pyknotic nuclei, nuclear de- o visible nuclear material. Subcap- as contained balloon cells and foci of coagulative necrosis (Fig. 3). A variable degree of congestion was also noticed.

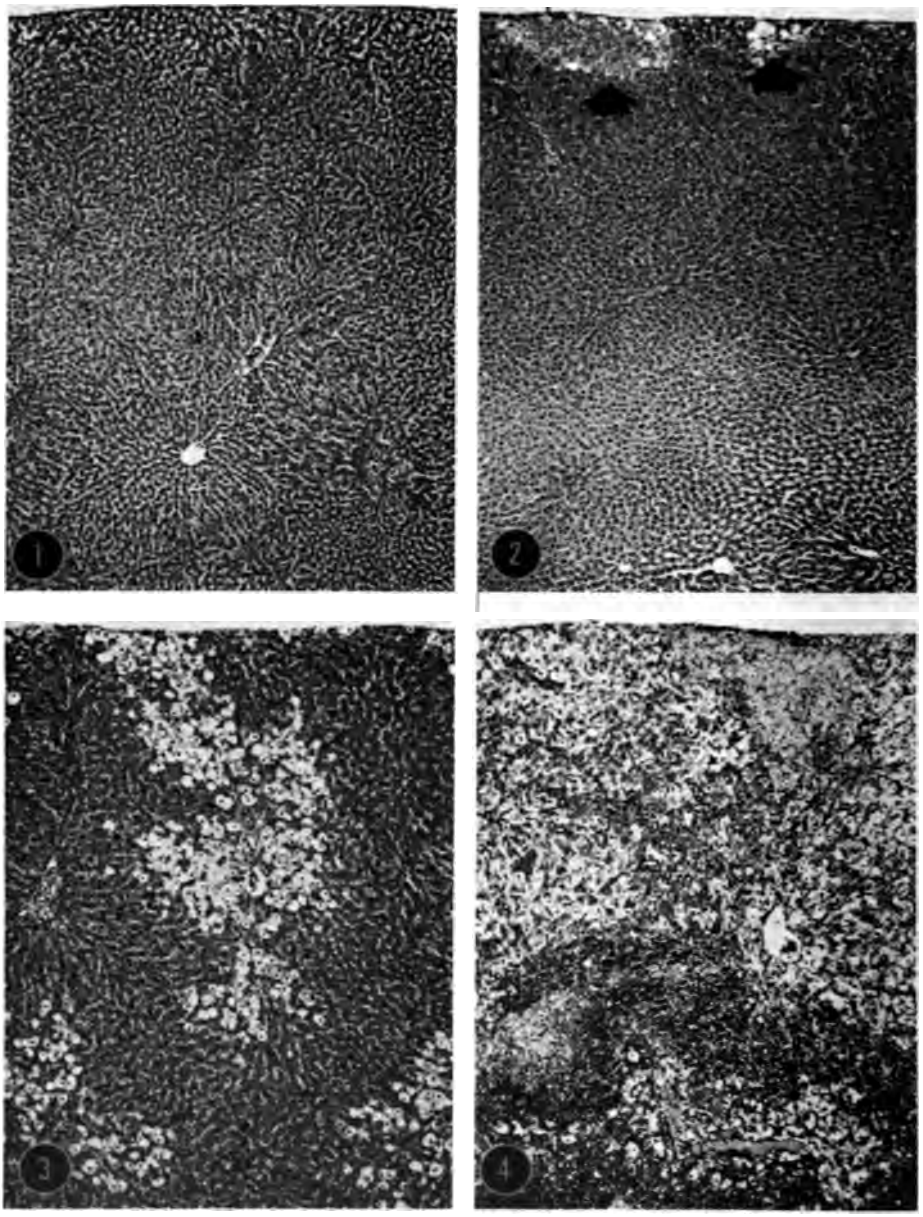
PCB + VDF (15,000 ppm). Animals in this group suffered extensive liver damage. Derangement of the hepatic architecture was complete, extending from the central vein to the portal triad. Ballooning of hepatocytes was detected even around the portal triad. Pale-staining necrotic tissue and large tracts of hemorrhage occurred in midzonal and centrolobular areas. The necrosis often involved the subcapsular regions (Fig. 4). Inflammatory cells were present in the necrotic tissue.

PCB + VDF (25,000 ppm). Livers of rats in this group were badly damaged, but not always clearly worse than those of the PCB + 15,000 VDF group. Massive areas of pale-staining necrosis involved the centrolobular and midzonal areas, frequently reaching the subcapsular spaces. Foci of inflammatory cells and hemorrhage bordered these necrotic areas. The periportal areas were filled with balloon cells (Fig. 8).

Discussion. Acute hepatic effects of exposure to VDF developed only in rats pretreated with PB or PCB (Table I), both of which induce the MFOS (9, 13). This dependence of acute VDF toxicity on pretreatment with MFOS inducers implies that metabolism of VDF to tissue-damaging metabolites is responsible for the toxic reaction. Metabolic activation of chlorinated ethylenes is generally believed to occur by epoxidation (14). The structural similarities of VDF with the family of chlorinated ethylenes suggest that an epoxide of VDF may also be formed. Such a reactive intermediate could be responsible for the acute hepatotoxicity reported here.

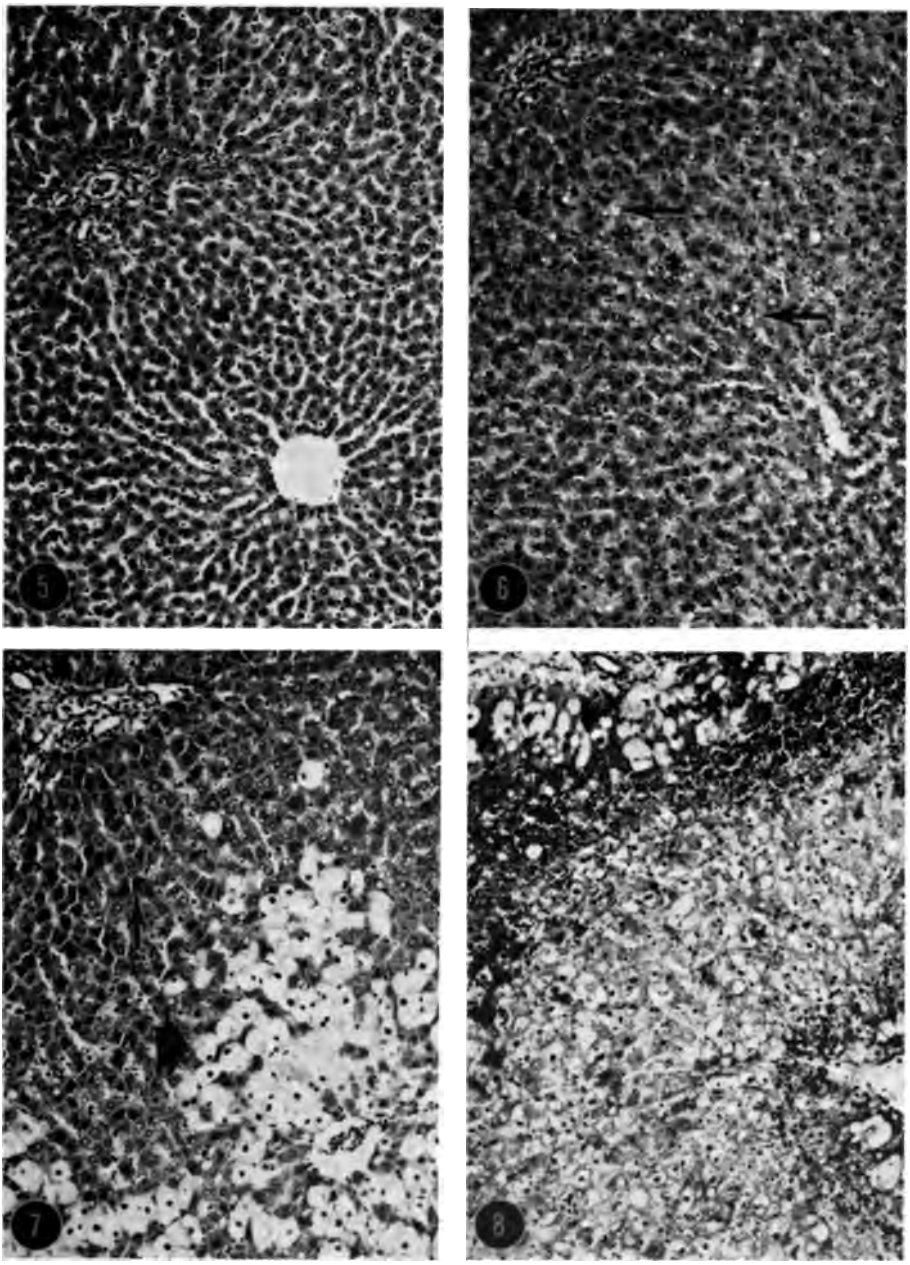
The potency of VDF as an acute hepatotoxin is roughly the same as that of a series of structurally related compounds—ethylene, vinyl fluoride, vinyl chloride, vinyl bromide—which are also acutely hepatotoxic only in rats treated with a MFOS inducer (8). In addition, the spectra of hepatic lesions, both gross and microscopic, caused by these compounds are very similar.

In contrast, the acute toxicity of VDF is very different from that of its halogenated analog, vinylidene chloride (VDC: 1,1-dichloroethylene). VDC is a potent acute hepatotoxin, having a 24-hr LC_{50} (the concentration which kills 50% of the exposed rats in



FIGS. 1–4 Hepatic capsule at top, hematoxylin and eosin. ×53.2. (1) Essentially normal architecture of liver c rats which received only VDF (25,000 ppm) exposure. (2) Two foci (arrows) of ballooned and necrotic liver cells i the subcapsular region (PB + VDF 25,000 ppm). (3) Ballooning of hepatocytes extends from the surface into th deep parenchyma (PCB + VDF 5000 ppm). Note that the liver damage is more severe than with higher concentratio of VDF (25,000 ppm) in PB-pretreated rats. (4) Almost uniform derangement of hepatic architecture; generalize ballooning, region of hemorrhagic necrosis (PCB + VDF 15,000 ppm).

24 hr) of 600 ppm in rats exposed for 4 hr (15). Pretreatment with MFOS inducers protects against, rather than enhances, the acute effects of VDC and the hepatic lesion which develops after VDC exposure is morphologi cally distinct from that caused by VDF (16 Jaeger *et al.* (17) have proposed that the VD metabolite chloroacetate undergoes a "leth



FIGS. 5–8 Central vein at lower right, portal triad upper left, hematoxylin and eosin. ×133. (5) The liver of rats posed only to VDF (25,000 ppm) appears normal at higher magnification as well. (6) PCB treatment caused fine cuolization (arrows) of hepatocytes in the centrolobular and midzonal areas. (7) Ballooning of hepatocytes orizontal arrow) in centrolobular space involving about half of the lobule. In other regions hypereosinophylic ark) hepatocytes with pyknotic nuclei are visible (vertical arrow) (PCB + VDF 5000 ppm). (8) Panlobular hepatic jury with hemorrhage around the pale, necrotic part and ballooning of hepatocytes around the portal triad (PCB VDF 25,000 ppm).

synthesis" in the citric acid cycle in a manner similar to that of fluoroacetate. This metabolic sequence for VDC involves a chloride shift as VDC epoxide rearranges to the corresponding chloroacyl chloride (18, 19). F–C bonds are stronger than Cl–C bonds and F migration during metabolism is less likely than is Cl migration (20). If the epoxides of both VDF and VDC are formed, the strikingly different toxicities of these two compounds may, therefore, be due in part to the differing potentials of the two epoxides to undergo rearrangements involving halogen migration.

PCB was much more effective than PB in sensitizing rats to the acute hepatotoxicity of VDF (Table I). This parallels the reports of Moslen *et al.* (21), Jaeger *et al.* (22), and of unpublished data from our laboratory which show PCB to be significantly more effective and potent than PB in sensitizing rats to the acute hepatic effects of ethylene, vinyl fluoride, and vinyl chloride. Alvares and Kappas (9) have shown PCB (Aroclor 1254) to be a "mixed type" inducer, having both PB and 3-methylcholanthrene-like effects on the MFOS. These data suggest that the toxic metabolite(s) of VDF is (are) generated by a specific enzyme or group of enzymes preferentially induced by PCB.

It is clear that VDF, ethylene, vinyl fluoride, vinyl chloride, and vinyl bromide share a number of characteristics: They are structurally similar; none is normally acutely toxic in humans or rats; their potencies as hepatotoxins are roughly similar; both the gross and microscopic appearances of the hepatic lesions they cause are similar; and their toxic effects in the rat are enhanced to a greater extent by PCB than by PB. These similarities suggest that a common mechanism may underlie the acute hepatic injury which results from PCB treatment with subsequent exposure to one of these compounds.

Summary. In rats pretreated with either phenobarbital (PB) or with polychlorinated biphenyl (PCB), an acute hepatotoxic reaction developed within 24 hr of inhalation exposure to vinylidene fluoride (VDF:1,1-difluoroethylene). PCB is much more effective than PB in this sensitization, which was evaluated by measurement of liver weight, serum sorbitol dehydrogenase activity, and by light-microscopic examination of the liver. PCB-treated rats received 5000, 15,000, or 25,000 ppm VDF for 4 hr while rats treated with PB inhaled 25,000 ppm VDF for 6 hr. The toxic effects of VDF in PCB-pretreated rats were dose responsive. These findings indicate that hepatotoxicity after VDF exposure can occur in rats and that these reactions (e.g., metabolism) can be stimulated by enzyme inducers. In light of the known activation of chlorinated ethylenes to epoxides, the analogous structures of VDF and the chlorinated ethylenes suggest that the epoxide of VDF may be part of the observed toxicity.

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Inhibition of Ovulation in Hamsters by the Protein Synthesis Inhibitors Diphtheria Toxin and Cycloheximide (40639)

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Pool and Lipner (1) reported that actinomycin D, an inhibitor of DNA-directed synthesis of messenger RNA (transcription), inhibited ovulation in rabbits. In this species, coitus triggers the release of pituitary luteinizing hormone (LH) into the blood stream and ovulation occurs about 11 hr later. These workers injected the inhibitor directly into ovarian follicles and observed inhibition of ovulation only by injections given up to 5 hr after mating (1). Similar results were obtained with puromycin and cycloheximide (2), inhibitors of mRNA-directed protein synthesis (translation). These findings led Pool and Lipner (2) to suggest that RNA and protein synthesis essential for ovulation is restricted to the early part of the 11-hr ovulatory process initiated by coital-induced LH release. Barros and Austin (3) reported that systemic injection of actinomycin D inhibited ovulation in the golden hamster, a spontaneously ovulating species. In this paper we report effects of systemic injection of two translational inhibitors, diphtheria toxin and cycloheximide, on ovulation in hamsters. Diphtheria toxin depletes cells of elongation factor 2, which is needed for growth of nascent peptide chains, by catalyzing its ribosylation by nicotinamide adenine dinucleotide (4). Cycloheximide prevents both peptide chain initiation and elongation by interacting with the 60S ribosomal subunit (5).

Materials and methods. Golden hamsters (*Mesocricetus auratus*) were used. In this species, ovulation occurs every fourth day and is photoperiodically controlled. When the laboratory is illuminated daily for from 12 to 16 hr, and when the midpoint of this photoperiod is used as the noon reference, the hypothalamic–pituitary system releases LH into the blood stream at about 2:30 PM on Day 4. The released LH stimulates the ovaries to secrete progesterone, which initiates behavioral estrus at about 5:00 PM, and further action of the LH causes follicular rupture (ovulation) about 1:30 AM of the next day (Day 1), i.e., about 11 hr after LH release. Therefore, drugs that block ovulation when injected before but not after 2:30 PM on Day 4 may be assumed to act on the CNS–pituitary system, whereas drugs effective after 2:30 PM may be assumed to act directly on the ovaries (6–12).

Hamsters from our own closed colony (FDA strain) or from the Lakeview colony (LVG:LAK strain) were used. They were fed Purina chow and water. The experimental room was illuminated daily from 4:00 AM to 8:00 PM E.S.T. (i.e., light–dark (LD) 16:8 with the midpoint of the light period synchronized with solar noon). FDA hamsters were born and raised in the experimental room. Lakeview hamsters were acclimated in it for at least 1 month before experimentation.

Materials were injected i.p. as a single dose in a volume of 5 ml/kg of body weight at specific times on Day 4, which was identified by daily analysis of vaginal washings (12). The hamsters were killed with ether or chloroform on the next day (Day 1) between 6:45 AM and 4:30 PM. Fallopian tubes were excised and ova were counted under a microscope at 100× as each tube was pressed separately between two glass slides. Data were recorded as the combined number of ova from both tubes of an individual hamster. Normally, 6–16 (mean value 12) ova are ovulated (6).

Diphtheria toxin was prepared as previously described (13) and had a potency of 8.8 guinea pig minimal lethal doses (MLD)/μg of toxin protein. Toxin was dialyzed at 4° in potassium phosphate buffer (0.01 *M*, pH 6.9) and bovine serum albumin was added to a concentration of 0.05%. The final solution, containing 570 μg of toxin/ml, was stored at −4° until the time of injection when it was diluted with buffer to the desired doses. Equine diphtheria antitoxin (Wyeth Co.,

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ı, Pa.) was diluted with the phos-
r. Cycloheximide (Acti-Dione,
Inc., San Diego, Calif.) and so-
barbital were dissolved in physi-
ıe.

he LD_{50} of diphtheria toxin in
s 6.5 μg/kg of body weight; con-
:s were 4.8–8.7 μg/kg. The MLD
/kg, or 1 μg/hamster. Since the
the sample was approximately
pig MLD/μg, it is estimated that
: nine times less sensitive to diph-
than are guinea pigs.

a toxin was found to be a potent
ovulation in hamsters. Data in
ıw that a dose of two hamster
g/kg) inhibited ovulation com-
hamsters when injected between
)0 PM on Day 4 of the estrous

EFFECT OF DIPHTHERIA TOXIN ON
ON IN HAMSTERS: TIME RESPONSE

'ubal ova counts on the day after injection[b]		
Toxin dose		
2 MLD[c] (22 μg/kg)	2 MLD + 200 units antitoxin/kg	Controls (5 ml buffer/kg)
, 0, 0, 0, 0, 0	10, 11, 11	
, 0, 0, 0, 0, 0	11, 11, 12	
, 0, 0, 0		
, 0, 0, 0, 0, 0		
, 0, 0, 2, 4, 9	7, 8, 11, 11, 13, 14	7, 9, 14, 15
, 0, 4, 10, 15, 16		
, 12, 12, 14, 14, 16		
, 9, 10, 11, 11, 13		

H is released into the circulation at about 4 and ovulation occurs about 11 hr later.
is the total number of ova found in both of an individual hamster. Normally 6 to 2) are ovulated.
: minimum lethal dose for a hamster.

cycle. Thereafter the toxin injection was gradually less effective, and was completely ineffective by 7:30 PM of the same day. Inhibition of ovulation by toxin was eliminated by diphtheria antitoxin, and thus it can be concluded that the blocking effect on ovulation is toxin specific. Dose–response data of the inhibitory effect of diphtheria toxin on ovulation are shown in Table II. The block in ovulation was found to be dose dependent. Ovulation was inhibited when the dose administered at 7:30 PM was increased to 4 and 8 MLD.

Cycloheximide also inhibited ovulation in hamsters (Table III). In lethality tests, doses of 80, 60, 40, and 20 mg/kg killed 4/4, 6/15, 1/8, and 0/6 hamsters within 1–6 days. Like the 2 MLD dose of diphtheria toxin, the 60 mg/kg dose of the drug inhibited ovulation in hamsters injected at 1:30 and 4:00 PM but not at 7:30 PM on Day 4. The 80 mg/kg dose of drug injected at 7:30 PM inhibited ovulation to the same extent as the 8 MLD dose of toxin (Table II). It is noteworthy that ovulation was inhibited by injection of cycloheximide as late as 10:45 PM, i.e., between 8 and 9 hr after LH release, and 2–3 hr before ovulation.

Although injection of the 60 mg/kg dose of cycloheximide or the 2 MLD dose of toxin at 1:30 or 4:00 PM on Day 4 inhibited ovulation, it did not inhibit behavioral estrus, as evidenced by an immediate display of lordosis by the treated female when exposed to a male at 7:30 PM on the day of injection or by the presence of sperm in the vaginal washing taken on the following morning.

That LH release occurred on Day 4 before 3:00 PM under the conditions of these experiments was confirmed by use of phenobarbital, an established central blocking agent for LH release (8, 11). Data in Table IV show that ovulation generally was inhibited by injection of phenobarbital at 1:30 or 1:45 but not at 3:00 PM, when injection of either toxin or cycloheximide was highly inhibitory. In a

BLE II. EFFECT OF DIPHTHERIA TOXIN ON OVULATION IN HAMSTERS: DOSE–RESPONSE

Tubal ova counts on the day after injection				
Controls (5 ml buffer/kg)	Toxin dose (MLD)			
	1	2	4	8
9, 9, 11, 12, 13, 16	0, 0, 0, 0, 0, 0, 1, 6	0, 0, 0		
	0, 0, 1, 2, 5, 9, 11, 12	0, 0, 0, 0, 5		
		7, 7, 10, 11, 11, 12	3, 7, 8, 8, 9, 9, 9	0, 0, 0, 0, 5, 6

TABLE III. EFFECT OF CYCLOHEXIMIDE ON OVULATION IN HAMSTERS

Time of i.p. injection on Day 4 of cycle (PM)	Tubal ova counts on the day after injection			
	Cycloheximide (mg/kg)			
	80	60	40	20
1:30	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 2	4, 7, 9, 10	9, 12
4:00	0, 0, 0, 0, 0, 0	0, 0, 0, 0		
6:00	0, 0, 0, 0, 6			
7:30	0, 0, 0, 3, 7, 7	5, 8, 10, 10, 12, 15		
8:30	0, 0, 1, 4, 8, 9			
10:45	0, 0, 0, 0, 1, 1, 6, 6, 8			

TABLE IV. EFFECT OF PHENOBARBITAL, DIPHTHERIA TOXIN, AND CYCLOHEXIMIDE ON OVULATION IN HAMSTERS

Time of i.p. injection on Day 4 of cycle (PM)	Tubal ova counts on the day after injection				
	Phenobarbital (140 mg/kg)	Diphtheria toxin (2 MLD)	Cycloheximide (80 mg/kg)	Solvent controls (5 ml/kg)	
				Saline	Buffer
1:30	0, 0, 0, 0, 0				
1:45	0, 0, 0, 0, 13				
3:00	0, 6, 9, 10, 11, 13, 14, 15	0, 0, 0, 0, 0, 0, 0, 15	0, 0, 0, 0, 0	9, 10, 11, 14	10, 12, 14, 15

previous report from this laboratory, the estimated time ± SE at which injection of phenobarbital blocked ovulation in 50% of a group of 346 hamsters exposed to LD 16:8 was found to be 2:30 PM ± 2 min (12), which is at the same time blood concentrations of LH rise on Day 4 in untreated hamsters exposed to LD 14:10 (9, 11).

Ovaries of hamsters providing tubal ova counts in Table IV were examined microscopically. The absence of tubal ova following injection of phenobarbital, toxin, and cycloheximide was associated with large unruptured ovarian follicles that, when pricked with a needle and observed at 30× magnification or pressed between two slides and observed at 100× magnification, released an oocyte surrounded by granulosa cells. In contrast, tubal ova were associated with large ruptured hyperemic follicles (corpora lutea) lacking oocytes. Other hamsters (two/group) were injected on Day 4 with phenobarbital (140 mg/kg) at 1:45 PM, diphtheria toxin (2 MLD) at 3:30 PM, or cycloheximide (80 mg/kg) at 3:30 PM and were killed on the following morning. None of the fallopian tubes contained ova. Ovaries were fixed in neutral-buffered formalin, serially sectioned, and stained with hematoxylin and eosin. Large unruptured and nonluteinized follicles with intact oocytes were observed in each group. Follicles of hamsters in which ovulation was inhibited by phenobarbital and cycloheximide were similar in the histologic appearance of their oocytes, and their theca and granulosa cells, which displayed various stages of mitosis. In contrast, in those in which ovulation was inhibited by toxin, the follicles contained shrunken oocytes and nondividing pyknotic granulosa cells in all phases of necrosis while theca cells appeared normal.

Discussion. The fact that ovulation was inhibited by injection of diphtheria toxin or cycloheximide after 2:30 PM on Day 4 of the estrous cycle, when release of LH from the pituitary gland into the blood stream occurs, indicates that these agents act directly on the ovaries. Since timing of the injection determines the described effects of ovulation, the effect is not secondary to the general inhibition of protein synthesis that culminates in death. The 2 MLD dose of diphtheria toxin and the 60 mg/kg dose of cycloheximide inhibited ovulation in virtually all hamsters injected up to 4:00 PM and in no hamster injected at 7:30 PM or later (Tables I–IV). Thus, the period of inhibition extends up to 5 hr after LH release. This period is remarkably similar to that obtained by Pool and Lipner (1, 2) after intrafollicular injection of actinomycin D, cycloheximide, and puromycin in rabbits (5–6 hr) and by Barros and Austin (3) after i.p. injection of actinomycin D in hamsters (4.5–6.5 hr, according to our analysis of their data). The latter workers exposed hamsters to light daily from 0800 to

hr (i.e., 5:00 AM–7:00 PM if the midpoint
: photoperiod is considered noon, as in
ıaper) and assumed LH was released at
hr (11:00 AM), when it is now known to
leased 3.5 hr later (6–12). Contrary to
ɔnclusion of Barros and Austin (3), there
ars to be no significant species difference
ɔen the rabbit and hamster in this early
d of susceptibility to actinomycin D.
, in both a reflex and a spontaneously
ıting species, injections of transcriptional
:ranslational inhibitors delimited an al-
identical period of inhibition of ovula-
extending with decreasing effectiveness
the first 5 hr of the 11-hr ovulatory
ɜss initiated by LH release. It is highly
ely that this singular effect resulted from
ns of these agents other than the one
have in common, i.e., inhibition of pro-
ɨynthesis.

ɔl and Lipner (1, 2) interpreted this ef-
ıs evidence that protein synthesis essen-
ɔr ovulation is restricted to the first few
ɜ after LH release. Although our results
the 2 MLD dose of toxin and the 60 mg/
ɔse of cycloheximide are consistent with
uggestion, we find that higher doses are
y inhibitory when injected at 7:30 PM
later (Tables II, III). The observation
ɔvulation can be inhibited by cyclohex-
: injected as late as 8–9 hr (10:45 PM)
LH release suggests that protein synthe-
tal for the process of ovulation continues
ıghout the 11-hr ovulatory period and is
estricted to the period immediately fol-
ıg LH release.

e present finding with diphtheria toxin
first case of a protein inhibiting ovula-
by direct action on the ovaries. The
heria toxin molecule resembles LH in
ining one subunit that combines with
fic receptors on the susceptible cell
ıa membrane and another subunit met-
cally active inside the cell (4, 14). How-
it is unlikely that the toxin inhibits
ıtion by occupying receptor sites for LH
varian cell membranes. Ovulation in
ters can be blocked by injection of LH
ɜrum on Day 4 at 1:00 PM but not at
ɔM (8). Apparently, LH binding to ovar-
ɜceptors is complete by 3:00 PM, and yet
ıtion was inhibited by diphtheria toxin
:ed more than 4 hr later.

Rondell (15) proposed that LH-induced ovulation involves two stages of protein synthesis: LH-induced steroidogenesis from acetate or cholesterol to progesterone, and progesterone-induced synthesis of collagenase in the ovarian follicle wall, causing its dissolution and culminating in ovulation. Strickland and Beers (16, 17) suggested that the lytic enzyme is not collagenase but plasmin, converted from plasminogen by the enzyme, plasminogen activator. In any case, the fact that diphtheria toxin and cycloheximide, as well as actinomycin D (3), inhibited ovulation but not behavioral estrus, which requires progesterone, favors the idea that these agents inhibit synthesis of enzymes responsible for dissolution of the follicle walls rather than enzymes involved in steroidogenesis.

Summary. Diphtheria toxin and cycloheximide, structurally unrelated translational inhibitors of protein synthesis, inhibited ovulation in hamsters. Inhibition resulted from i.p. injection given well after the 2:30 PM release of pituitary luteinizing hormone (LH) that initiates the 11-hr ovulatory process. Thus it may be concluded that these agents inhibit ovulation by direct action on the ovaries. Both luteinization and follicular rupture were inhibited. Follicles inhibited by cycloheximide were similar histologically to control follicles prevented from ovulating by central blockade of LH release with phenobarbital, whereas follicles inhibited by toxin contained shrunken oocytes and necrotic granulosa cells. Low doses of both agents inhibited ovulation only when injected within 5 hr after LH release. However, high doses were effective at later times. Similar ovulation inhibition responses to both the microbial toxin and cycloheximide at high doses suggest that protein synthesis essential for ovulation occurs throughout the 11-hr ovulatory process initiated by LH and is not restricted to the early part of this period.

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ıymidine Phosphorylase Activity in Plasma: A Cancer Marker or an Artifact of Ultrafiltration? (40640)[1]

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midine phosphorylase (EC 2.4.2.4, thy-
:orthophosphate deoxyribosyltransfer-
atalyzes the reversible reaction: thy-
: + orthophosphate ⇋ thymine +
:y-α-D-ribofuranose-1-phosphate (1).
ızyme is present in many normal and
ıstic eukaryotic cells (1–3) and is found
ı prokaryotes (1, 4).
uman subjects, thymidine phosphoryl-
tivity is present in a variety of tissues
including the leukocytic fraction of
obtained from both normal subjects
atients with leukemia (5). Recently,
er, it was reported that thymidine
horylase activity is present also in a
e plasma fraction obtained from nor-
ubjects (6), as well as from patients
alized with neoplastic diseases, some of
had received chemotherapy (7–9). In
ter group, the reported mean level of
line phosphorylase activity in 40 μl of
a (52.7 ± 17.6%, as percentage degra-
of thymidine ± SD) was approxi-
/ twofold higher than that determined
: healthy donors (23.9 ± 13.7%). It was
ed, therefore, that an elevated level of
zyme activity could be used as a diag-
marker in the detection of certain
of cancer (7).
ent studies on the distribution and sub-
specificity of various pyrimidine nu-
le phosphorylase activities (P. W.
man, unpublished data) have demon-
l that the thymidine phosphorylase ac-
of normal human blood is located pri-
/ in the granulocyte fraction, a finding
consistent with earlier observations by
and Perry (10), using less purified prep-
ns of granulocytes and lymphocytes.
: absence of thymidine phosphorylase
y from the plasma (2.9 ± 0.2%, per-
centage degradation of thymidine (80 pmole) per 25 μl of plasma ± SD) was demonstrated by the use of procedures that prevented either lysis of cells or contamination of the plasma with granulocytes, or both. Accordingly, the method used by Pauly and co-workers (6–9) to obtain their cell-free plasma fractions was examined, to determine whether or not it was responsible for an artifactual localization of thymidine phosphorylase activity in the plasma.

Evidence is presented to show that thymidine phosphorylase activity is not usually present in plasma from healthy subjects, but only appears there when the technique used to fractionate the blood causes either cell lysis or contamination of the plasma fraction with granulocytes or both.

Materials and methods. Fractionation of plasma. All chemicals used were of analytical grade. Twenty milliliters of human blood were collected in heparinized syringes from each of nine healthy subjects. The blood was centrifuged at 500*g* for 12 min; the plasma supernatant fraction (A) was removed, divided in half, and stored on ice until processed. One-half of the plasma fraction (A) was centrifuged at 10,000*g* for 30 min at 4°; the supernatant fraction (B) was harvested and the cell pellet (C) obtained was resuspended in a 20 m*M* sodium phosphate/10 m*M* β-mercaptoethanol buffer, pH 8.0 (Buffer 1),[2] and the cells were lysed by freeze/thawing. An aliquot of the supernatant material (B) was saturated to the level of 55% with ammonium sulfate (Schwarz–Mann, Orangeburg, N.Y.) at 4°. The precipitate was harvested by centrifugation at 16,000*g* for 20 min at 4° and the clear supernatant fraction was discarded, while the pellet (D) was resuspended in Buffer 1 and dialyzed against the

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[2] Abbreviation used in this paper: Buffer 1:20 m*M* sodium phosphate/10 m*M* β-mercaptoethanol, pH 8.0.

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same buffer for 4 hr with four changes of buffer.

The remaining half of the plasma (A) was processed according to the method used by Pauly and co-workers (6–9); thus, fraction (A) was filtered through a 0.22-μm membrane (Millipore Corp., Bedford, Mass.) to remove cells and debris, which yielded a filtrate free of cells (but not the products of their earlier rupture). Because of the number of cells still present in fraction A, the 0.22-μm membrane rapidly became clogged; thus, it was not possible to filter more than 150 μl of plasma with one filter. To obtain a larger volume of cell-free plasma (E), it was necessary to recentrifuge the plasma supernatant material (A) at 800*g* for 10 min before filtering it through the 0.22-μm membrane. An aliquot of E was saturated to the 55% level with ammonium sulfate at 4°, and the precipitate (F) was harvested and treated as described for D.

Samples were taken at each stage of the fractionation and differential blood cell counts made on smears stained with Wright's solution. Fractions A to F were stored at −22° until assayed. Protein levels were determined by the method of Lowry *et al.* (11).

Thymidine phosphorylase assays. Thymidine phosphorylase activity was assayed in the catabolic direction, using two types of conditions. Assay 1 employed essentially the conditions of the microassay developed by Pauly *et al.* (6–9), while Assay 2 utilized saturating substrate conditions. In Assay 1, 40 μl of the plasma fraction were added to 60 μl of RPMI medium 1640 (pH 7.2) containing 1.6 μCi [*methyl*-^3H]thymidine (Amersham/Searle, Arlington Heights, Ill.: sp. act., 24 Ci/mmole).

For Assay 2, the 100-μl incubation mixture contained 0.1 *M* sodium phosphate buffer at pH 6.4, β-mercaptoethanol (2.5 m*M*), and 2.0 μCi [*methyl*-^3H]thymidine (Amersham/Searle, sp. act. 24 Ci/mmole), 25 μl of the plasma fraction, and 1.6 m*M* unlabeled thymidine (Sigma Chemical Co., St. Louis, Mo.). The mixtures were incubated at 37° for 3 hr and the reaction was terminated by boiling for 2 min. [^3H]Thymine and [^3H]thymidine were separated from each other on silica gel uv_{254} polygram plates (Brinkman, Westbury, N.J.), using chloroform:methanol (9:1). Radioactivity was measured in a liquid scintillation counter (Amersham/Searle MK III). The results obtained are expressed as either picomoles of thymidine cleaved per milligram of protein, or as the percentage degradation of thymidine (6–9). Student's *t* tests were performed, using a formula modified for unequal variances (12).

Results. As shown in Table I, the highest levels of thymidine phosphorylase activity were detected in the lysates of the pellets (C) (composed predominantly of white blood cells, derived from the centrifuged crude plasma of each of the nine subjects studied) by use of both Assays 1 and 2. In contrast, the two cell-free plasma fractions, B and E, both contained relatively low levels of thymidine phosphorylase activity (Table I).

High-speed centrifugation of the initial "plasma" fraction A significantly decreased the level of enzyme activity present; thus, the plasma supernatant fraction obtained at

TABLE I. DISTRIBUTION OF THYMIDINE PHOSPHORYLASE ACTIVITY DURING THE FRACTIONATION OF CELL-FREE PLASMA FROM NORMAL HUMAN BLOOD[a]

Fraction	Assay 1	Assay 2
(A) Plasma supernatant fraction from whole blood (centrifuged at 500*g* for 12 min)	4.6 (±1.8)[b]	6.9 (±3.2)
(B) Cell-free plasma supernatant fraction from (A) (centrifugation at 10,000*g* for 30 min, 4°)	0.6 (±0.5)	1.6 (±0.8)
(C) Lysate of the pellet formed from (A) by (B)	490.0 (±399.0)	476.7 (±384.7)
(D) 0–55% ammonium sulfate "cut" of (B)	3.0 (±3.3)	1.8 (±0.4)
(E) Ultrafiltrate of plasma supernatant fraction from (A) (800*g* for 10 min)	1.4 (±0.6)	2.7 (±1.2)
(F) 0–55% ammonium sulfate "cut" of (E)	6.3 (±3.2)	5.7 (±3.3)

[a] Twenty milliliters of blood were collected from each of nine normal subjects and fractionated as described under Materials and Methods. Fractions were assayed in duplicate for thymidine phosphorylase activity, using both Assays 1 and 2. Values are the mean of those determined for each of the nine subjects ± the standard deviation (SD).

[b] pmole of thymidine cleaved/mg protein (±SD).

DIFFERENTIAL CELL COUNT OF LEUKOCYTE NTS PRESENT IN THE PLASMA SUPERNATANT FRACTION A[a]

lo.	% Granulocytes	% Lymphocytes/ Monocytes
	49	51
	51	49
	60	40
	64	36
	52	48
	72	28
	67	33
	52	48
	75	25

n A was obtained by centrifuging 20 ml of an blood for 12 min at 500g. Smears were the supernatant fractions and stained for cell counts.

B), which was completely free of ained levels of thymidine phospho- ivity that were about one-eighth (P nd about one-fourth ($P < 0.002$) of ginally present in the plasma pre- 500g (A), as determined by Assays respectively. As shown in Table I, of the cellular contaminants from A eed centrifugation, coupled with ul- on, in the manner described by *al.* (6–9), only lowered the level of phosphorylase activity of crude A) to about one-third (E), as meas- Assays 1 and 2 ($P < 0.001$ and $P <$ ectively); consequently, the level of ctivity in the latter type of plasma E), was about twice that ($P < 0.02$) the cell-free plasma fraction (B) by high-speed centrifugation.

vels of thymidine phosphorylase ac- ne fractions obtained by ammonium recipitation, expressed as the per- egradation of thymidine (± SD), as ed by Assays 1 and 2, respectively, ±2.1)% and 3.1 (±0.9)% for fraction 2 (±2.7)% and 6.0 (±3.0)% for frac- These values represent an approxi- bling ($P < 0.002$ and $P < 0.001$, ly) of the thymidine phosphorylase a cell-free plasma filtrate E, as com- hat present in fraction B.

sults in Table II clearly show the of leukocytes, especially granulo- he initial plasma fraction A. Similar re obtained with fraction C (the cell small number of erythrocytes and platelets also were present in fractions A and C, although the data are not tabulated. Previous work, however, has shown that normal red blood cells (1, 5) and platelets (P. W. Woodman, unpublished data) do not contain detectable levels of thymidine phosphorylase activity. There were no cells or debris present in either the cell-free plasma fractions B and E or their respective ammonium sulfate fractions D and F.

Discussion. The data in Tables I and II are consistent with findings that the thymidine phosphorylase activity of blood of healthy donors is located primarily in the granulocytes ((10) and P. W. Woodman, unpublished data) and ordinarily is not detected in the plasma in significant amounts, irrespective of either the assay conditions used (i.e., either Assays 1 or 2) or the way in which enzyme activity is expressed (either percentage degradation of thymidine or picomoles of thymidine cleaved per milligram of protein).

The plasma B, prepared from A by high-speed centrifugation, contained no cellular contaminants; hence, the extremely low levels of thymidine phosphorylase activity present may have come from lysis of the granulocytes in fraction A, produced by low-speed centrifugation (500g for 12 min) of the whole blood. In addition, there is the possibility that the granulocytes had released some of their thymidine phosphorylase activity into the blood prior to fractionation (5, 13). On the other hand, the cell-free plasma fraction produced by low-speed centrifugation and ultrafiltration, E, contained a twofold higher level ($P < 0.02$) of thymidine phosphorylase activity than that of fraction B (produced by high-speed centrifugation (10,000g) for 30 min). Treatment of the two cell-free plasma fractions with ammonium sulfate also increased the thymidine phosphorylase activity to a more significant level with fraction E than was the case with fraction B. It is suggested, therefore, that the granulocytes present in the original plasma (A) were ruptured during the procedures employed by Pauly *et al.* (6–9), i.e., ultrafiltration through a 0.22-μm membrane, resulting in the release of their thymidine phosphorylase activity into the "plasma filtrate" (E).

Studies by others have shown that the levels of thymidine phosphorylase activity are

higher in normal human leukocytes than in those obtained from patients with chronic myelogenous leukemia (5, 10) or with either acute or chronic lymphocytic leukemia (5). In view of these findings (5, 10), as compared to those of Pauly *et al.* (9), who reported an elevated level of thymidine phosphorylase activity in the "plasma" they obtained from a patient with chronic myelogenous leukemia, it will be interesting to reevaluate the level of enzyme activity in a cell-free plasma fraction prepared without the use of ultrafiltration.

Because polymorphonuclear leukocytes can selectively release their lysosomal enzymes into the plasma (14), it is conceivable that intact leukocytes could also release traces of thymidine phosphorylase activity (5, 13). The present evidence strongly suggests, however, that the reported presence of thymidine phosphorylase activity in the plasma (6–9) may be an artifact of the method of its preparation, i.e., the rupture of granulocytes during the process of ultrafiltration.

Summary. The possibility was investigated that thymidine phosphorylase activity is released from leukocytes during ultrafiltration of crude "plasma," thus accounting for reports of this enzyme in "cell-free" plasma. Blood samples from nine healthy donors, when centrifuged at only 500*g* for 12 min, yielded plasma fractions that contained cells identified as leukocytes, erythrocytes and platelets, as well as significant levels of thymidine phosphorylase activity. Centrifugation of the crude plasma at 10,000*g* for 30 min produced a cell-free plasma that contained negligible levels of thymidine phosphorylase activity. By contrast, the sediment, which contained identifiable granulocytes, possessed very significant levels of enzyme activity. Centrifugation of the crude plasma at 800*g* for 10 min, when followed by ultrafiltration, yielded a cell-free plasma that contained a twofold higher level of thymidine phosphorylase activity ($P < 0.02$) than did the truly cell-free plasma produced by high-speed centrifugation.

Our findings substantiate earlier reports, which had indicated that thymidine phosphorylase activity is located primarily in the granulocytes, and suggest that significant levels of the enzyme ordinarily do not occur in true plasma obtained from normal blood, unless the granulocytes are ruptured. Consequently, the proposal that elevated levels of thymidine phosphorylase activity in plasma can be used as diagnostic markers for certain neoplastic states requires reevaluation.

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celerated Removal of Platelets during Perfusion of Isolated Lungs with Perfluoro Erythrocyte Substitute (40641)[1]

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high solubility of oxygen and carbon le in some liquid fluorochemicals (per- compounds) has permitted their use as breathing media for small animals (1). in of these compounds, when dispersed aqueous medium into particles smaller erythrocytes, have successfully carried e gas transport functions of the eryth-. This was first demonstrated in exper- s with an isolated perfused rat brain (2) it was found that the metabolic and ical activity (EEG) of the brain were ained as well by perfusion with emul- fluorochemical as with erythrocytes, at perfusion with plasma alone resulted id loss of electrical activity (3). Subse- ly, the ability of the dispersed perfluoro ounds to deliver oxygen to tissues of animals was demonstrated (4–6). Al- h it was clear that the dispersed per- compound transported oxygen *in vivo*, ection into the circulating blood was imes followed by derangements of cir- ry and pulmonary function (7, 8). In- ious injection of emulsified fluorochem- used a large decrease in the number of ating blood platelets (9) and in some ls, there were gross lesions in the lungs marked increase in resistance to flow od through the lungs (10). Since these s might be due to an action of fluoro- ical on lungs and/or blood platelets, we d the effects of adding emulsified fluo- mical to the fluid perfusing isolated when blood platelets were either pres- r absent from the fluid. Our results indicate that the fluorochemical did not have a direct, deleterious effect on the lungs. The accelerated removal of platelets from the perfusion fluid which occurred suggests that there was an interaction between fluorochemical particles and platelets which resulted in deposition of platelets in the lungs.

Materials and methods. Emulsions of FC-80 (perfluorobutyltetrahydrofurane, 3M Co.) were freshly prepared for each experiment by sonication in an 8% solution of bovine serum albumin (Fraction V, Sigma Chem. Co.) in Krebs–Ringer bicarbonate buffer, as previously described (3). The final concentration of the fluorochemical in the emulsion was approximately 40% (v/v), which is about 80% (w/v) since the density of the fluorochemical is nearly 2. The particles were 1 μm in diameter and the pH of the emulsion was 7.35.

Male guinea pigs (Hartley strain) or male rats (Sprague–Dawley strain) weighing about 300 g, were anesthetized with pentobarbital and ventilated mechanically through a tracheostomy. After a catheter was placed in the pulmonary artery, the lungs were removed and suspended in a water-jacketed chamber maintained at 37°. The pulmonary artery was perfused at 10 ml/min with recirculating fluid consisting of 4% bovine serum albumin and 5 m*M* glucose in Krebs–Ringer bicarbonate buffer (pH 7.4) which was aerated with 5% CO_2 in oxygen. The lungs were ventilated with the same gas mixture at a rate of 80 per min and a tidal volume of 2.5 ml. The lung preparation, with its connecting tubing, was suspended by three wires from an analytical balance (Mettler Model P163) and weighed continuously. A weight change of 10 mg (0.5% of the weight of the lungs) was reliably detectable. The pressures required for lung ventilation and perfusion were monitored by strain gauge transducers and recorded continuously. The pH of the perfusion fluid was monitored continuously with a flow cuvette

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and glass electrode and the pH was maintained between 7.35 and 7.45 by addition of either sodium hydroxide or hydrochloric acid solution. The technique of perfusion has been described previously (11, 12).

Blood platelets were obtained from human blood which had been collected in 3.5% sodium citrate (1 to 9 vol of blood). Human, rather than rat, platelets were used because we were interested specifically in the interaction of human platelets with emulsified fluorochemical. Our control experiments indicated that human platelets and rat lungs were apparently compatible. Platelet-rich plasma was prepared by centrifugation of the blood in plastic tubes at 200*g* for 10 min at room temperature. Platelets were counted by the method of Brecher and Cronkite (13).

Results. In one group of experiments, isolated lungs were perfused with fluid which contained no platelets. Emulsified FC-80 was absent (controls) or was added. The pressure required for tidal ventilation was approximately 5 to 7 cm of water and the mean perfusion pressure (with reference to the top of the lung) was 12 to 18 cm of water. Lung preparations perfused for 60–75 min without fluorochemical (controls) showed no significant changes in perfusion pressure or ventilation pressure and the weight of the lungs increased less than 5% of the initial weight. In five experiments (two rat and three guinea pig lung preparations) emulsified FC-80 was added to the perfusion fluid to give a concentration in the range of 5–10% (v/v). This concentration of emulsified fluorochemical when injected into the circulation of intact animals, has been previously shown to cause definite changes in pulmonary circulation and in the lungs (8, 9). In each of the five perfusion experiments, the addition of dispersed FC-80 caused no change in ventilation pressure and only a slight increase (<2 cm water) in perfusion pressure. Similar slight increases in perfusion pressure were observed when a suspension of washed erythrocytes was added to the perfusion fluid, suggesting that the slightly increased pressure was due to increased viscosity of the perfusion fluid rather than to a change in the lung. The weight of the lungs increased by less than 5% after 60–75 min of perfusion.

In another group of experiments, rat lungs were perfused in the same way but 30 min before addition of emulsified FC-80, human platelet-rich plasma was added to the perfusion fluid to yield a concentration of approximately 200,000 per mm^3. In these experiments, as in those done without platelets, there were no significant changes in pressures required for ventilation or pulmonary perfusion after addition of FC-80 emulsion to the perfusion fluid. Platelet counts were made at intervals after addition of platelet-rich plasma and after the subsequent addition of emulsified FC-80. In two control experiments, the emulsified FC-80 was omitted. In two additional control experiments both platelets and emulsified FC-80 were added but the lungs were omitted from the circuit in order to evaluate the loss of platelets during perfusion by deposition outside the lungs. The changes in the platelet counts during 60 min of perfusion are shown in Fig. 1. In two experiments without the lungs in the circuit, an average of 94% of the original platelets remained after 30 min. This suggests that a slow rate of platelet removal during lung perfusion was due primarily to platelet dis-

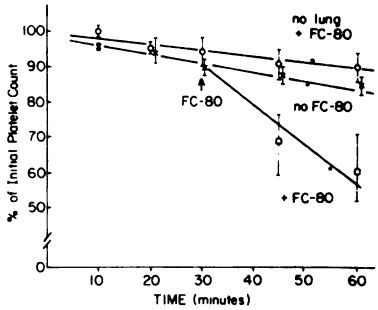

FIG. 1. The effects of emulsified fluorochemical on the rate of removal of human platelets from the fluid perfusing isolated rat lungs. The volume of recirculating perfusion fluid was 80 ml. In all experiments, 5 ml of a human platelet suspension was added at time zero, yielding an initial platelet count of approximately 200,000 per mm^3. The curve designations are: no lung + FC-80 for control experiments where the lungs were omitted from the perfusion circuit but FC-80 was added at the time indicated; no FC-80 for control experiments where the perfusion circuit was complete but FC-80 was not added; +FC-80 for experiments where the perfusion circuit was complete and FC-80 was added at the time indicated. In the experiments with FC-80, 10 ml of an emulsion (40% v/v) was added and the concentration of FC-80 in the perfusion fluid after mixing was 8% (v/v). The values shown are the average and the range of values.

or adherence to elements of the per-
ircuit, with only a small contribution
he presence of the lung itself. The rate
let loss was not significantly changed
rfusion up to 60 min in the absence of
emulsion, nor by the addition of FC-
lsion in the absence of the lung. The
emoval of platelets from the perfusion
as significantly accelerated at both 15
min after addition of the FC-80 emul-
ien the lungs were present (Fig. 1).
ologic examinations of rat lungs after
of perfusion with the FC-80 emulsion
slight perivascular "cuffing" with
milar to that observed in control lungs,
other abnormalities were noted by
icroscopy.

ussion. In our experiments, the lungs
erfused at a constant rate and venti-
t a constant tidal volume. Therefore,
s in the lung tissue, its airways, or its
ion would have led to changes in ven-
or perfusion pressure as previously
by the large changes in pressures after
n of 5-hydroxytryptamine (11) or with
oment of pulmonary edema (12).
ore, the observed lack of change in
on and ventilation pressures after ad-
of the fluorochemical in the present
ndicates that the pulmonary vascular
ice and mechanical properties of the
ere essentially unchanged during the
of perfusion. The absence of signifi-
eolar edema was also indicated by the
e of significant increase in lung weight
histologic examination. These results
that the fluorochemical emulsion did
e a direct toxic effect on the isolated
d lung. On the other hand, our results
that the emulsified fluorochemical
ted with blood platelets in a way that
d in their increased rate of removal.
ldition of emulsified FC-80 did not
in an increased rate of removal of
s in the absence of the lung, suggesting
e platelets (aggregated and/or other-
anged) were removed in the pulmo-
rculation. The lesions which occur in
gs of an intact animal after infusion of
ied fluorochemical might be caused
ries of events which starts with pul-
deposition of platelets. This possibil-
pported by the observations (9, 10) of
l reductions in circulating platelets *in vivo* and significant changes in coagulation factors (9) in animals infused with fluorochemical emulsions. Although there was not an increase in pulmonary vascular resistance in the perfused lungs after addition of fluorochemical to the platelet-containing perfusion fluid, the difference between this situation and the intact animal may be due to the absence of coagulation factors in the fluid perfusing the isolated lungs.

Recent work (14) has shown that putting a coating of lipid on the emulsified particles prevents some of the effects of FC-80 on blood platelets. This finding may lead to the development of a fluorochemical emulsion which does not cause lesions in the lungs and is safe for use as a blood substitute in intact animals.

Summary. An emulsion of a fluorochemical (FC-80), which has been shown to function as a substitute for the erythrocyte in the transport of O_2 and CO_2, has been found to cause lung damage when infused intravenously in intact animals. Addition of this emulsion to the fluid perfusing rat and guinea pig isolated lung preparations caused no significant changes in the pressures required for ventilation and perfusion of the lungs at constant flow rates, or in the weight of the lungs. Addition of this emulsion after prior addition of human blood platelets, caused a large increase in the rate of removal of platelets from the perfusion fluid. These results indicate that the fluorochemical emulsion does not have a direct deleterious effect on the lungs but that an interaction between fluorochemical particles, blood platelets, and the lung occurs, which might lead to lung damage.

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ıe Effect of Parathyroid Hormone upon Release of Degradative Enzymes from Rat Bone (40642)[1]

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ırathyroid hormone action on bone in- ɛs the mobilization of both calcium and phate and the resorption of organic ma- (1, 2). Many of the biochemical events h accompany these two effects of the ıone are recognized (3–5) but their time ence is unclear and the role of some of ɔmponents of normal bone matrix has ɔeen elucidated. Specifically, lipids have recognized as being part of the normal ıical makeup of bone matrix for some (6, 7). While some progress has been e in understanding the association of : lipids with bone formation (8) and min- transformation (9), there still appears to question as to their function. In an npt to add information with regard to question, the following series of experi- ts were carried out.

aterials and methods. All experiments carried out on specimens from 10-day- nale Wistar rats (body wt 19.5 ± 1.2 g).

ɛtermination of phospholipids. In order to tain which phospholipids were degraded arathyroid hormone, the following pro- res were carried out:

roups of five animals were sacrificed by pitation. The tibia were removed imme- ly after sacrifice and were dissected free ft tissue and periosteum. The bone mar- was removed by flushing with cold saline the metaphyseal portions were separated pooled. The pooled metaphyses of the l femora and proximal tibiae were incu- l in 5 ml of modified BGJB medium aining 2 units of purified bovine parathy- hormone (Eli Lilly Co.) per milliliter in ıbnoff incubator under 95% oxygen, 5% for 4 hr. A control group without para- ɔid hormone was incubated simultane- /. After incubation, the bones were ıed with distilled water three times and lyophilized. The lipids were extracted according to the method of Folch *et al.* (10). The total lipid phosphorous of the lyophilized bone was determined according to the method of Marinetti (11) and the phospholipid content was calculated by multiplying the phosphorous content of the lipid extract by 25 (11). Individual phosphatides were determined by thin-layer chromatography separation. Thin-layer plates were prepared from silica gel H (Sigma Chemical Co.) and stored in a desiccator over anhydrous $CaSO_4$. The plates were activated at 100° immediately before use. Chromatograms were developed with chloroform–methanol–acetic acid–water (24:14:4:2 by volume) (12). After development, the entire area was exposed to iodine vapor to visualize the separated lipids and the phosphatides were scraped from the thin-layer plate. There were three extractions of the phospholipids with a mixture of ethanol/chloroform/water/acetic acid (100:30:20:2). The lipid phosphorus was determined according to the method of Marinetti (11). Because phosphatidyl choline showed a larger percentage loss than phosphatidyl ethanolamine or sphingomyelin (see Results), it was selected as the substrate for enzyme assays.

Enzyme activity. The metaphyseal portion of one tibia was incubated in 1.0 ml of modified BGJB medium containing 2 units of parathyroid extract and 0.05 ml of ether solution containing labeled phosphatidyl choline ([*methyl*-^{14}C]choline) (New England Nuclear, Boston, Mass.) and 1 mg of unlabeled phosphatidyl choline purified from commercially available egg lecithin. Control values were obtained by incubation without parathyroid hormone. Incubations were carried out at 37° with continuous shaking for times varying between 2 and 48 hr. At the end of the reaction, the lipids in the medium were extracted with chloroform/methanol three times. After extraction, the aqueous layer was centrifuged, following which 0.2 ml of the

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of the aqueous solution was counted in a liquid scintillation counter.

Collagenolytic assay. Collagenolytic activity was assayed by measuring the release of soluble radioactivity from purified reconstituted collagen labeled with [^{3}H]proline and [^{3}H]hydroxyproline according to the method of Kaufman *et al.* (14). The reaction mixture consisted of 100 μl of the ^{3}H-labeled collagen gel formed by warming to 37° for 20 hr (approx 4000 cpm) in 400 μl of a modified BGJB medium containing 2 units of parathyroid extract and one tibial metaphysis from a 10-day-old rat. The mixtures were incubated for periods varying from 2 to 48 hr at 37°. Control values were determined by incubation without hormone. Blank values were obtained by parallel incubation using heat-killed tibial metaphysis. After completion of the incubation, the cultures were centrifuged at room temperature, a 200-μl aliquot of supernatant of each culture was dissolved in 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and counted in a liquid scintillation counter.

Determination of ^{45}Ca release. Six animals were injected subcutaneously with 2 μCi of ^{45}Ca. The animals were sacrificed by decapitation 20 hr later and the tibial metaphyses prelabeled with ^{45}Ca were dissected free of soft tissues and placed in 1.0 ml of a modified BGJB medium in pairs. One tibia was placed in control medium while the opposite tibia of the same rat was in medium supplemented with 2 units of purified parathyroid extract. Both plates were incubated at 37° at times varying from 2 to 48 hr after which 0.1 ml of the medium was dissolved in 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and the radioactivity was determined in a liquid scintillation counter. Statistical analysis of results was carried out using Student's *t* test.

Results. As can be seen in Fig. 1, there was a statistically significant increase in the release of ^{45}Ca into the medium by bones incubated with PTH as compared to those without the hormone. This became statistically significant ($P < 0.05$) at 4 and 10 hr and remained significant ($P < 0.01$) at 24 and 48 hr. As is also seen in Fig. 1, there was a parallel increase in the radioactivity from [^{14}C]phosphatidyl choline with PTH again leading to an increase in the amount of radioactivity measured. This difference was statistically significant ($P < 0.01$) at 4 hr and remained so for the duration of the experiment. Figure 2 demonstrates that the degradation products from labeled collagen were not found until 5 hr after the administration of the hormone and parathyroid hormone did not appear to increase this until much later than calcium release had been noted and phospholipid breakdown had been measured. The differences were statistically significant at 24 hr ($P < 0.05$) and 48 hr ($P < 0.01$). Table I indicates that following 4 hr of incubation, a statistically significant decrease in total phospholipid was seen in bone. There was a statistically significant difference in the amount of phosphatidyl choline and while there was a diminution in phosphatidyl ethanolamine and sphingomyelin, this was not statistically significant.

Discussion. The sequence of events whereby parathyroid hormone mobilizes serum calcium and leads to matrix resorption is still not clear. The eventual outcome is

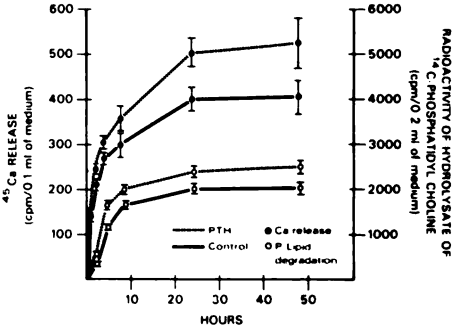

FIG. 1. The release of ^{45}Ca from prelabeled bones and of the degradation products from [^{14}C]phosphatidyl choline. Figures refer to time release and the standard error is shown. Each point is based on six determinations carried out in duplicate.

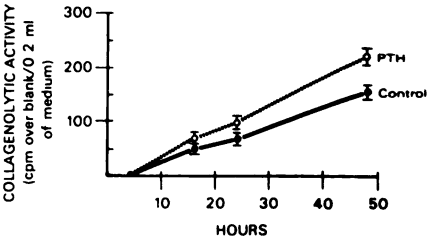

FIG. 2. The release of [^{14}C]proline and hydroxyproline from prelabeled collagen. Figures refer to time release and the standard error is shown. Each point is based on six determinations carried out in duplicate.

EFFECT OF PARATHYROID HORMONE ON LIPIDS OF RAT BONE AFTER 4 HR OF INCUBATION[a]

	Total phospho-lipid	Phospholipids mg/g bone[b]		
		PC	PE	SM
	11.8	5.8	2.5	1.1
	±0.9	±0.5	±0.4	±0.2
ml)	9.5*	4.2*	2.1	0.8
	±0.8	±0.6	±0.3	±0.2

e given as the mean ± SE of five deter-
: animals were used in one group and four
ones used in one pooled sample). Signifi-
:s between control and parathyroid-sup-
ıbations are indicated by: * $P < 0.01$.
)hatidyl choline, PE: phosphatidyl etha-
sphingomyelin.

both the organic and inorganic of bone but this is apparently -wise fashion. *In vitro*, the organic :ars to be resistant to digestion been at least partially demineral- his suggests a temporal sequence release, if indeed enzymes are ith early calcium mobilization. It ostulated that the early calcium ı is due to the production of lactic l citric acid (16–18) which is ca- ssolving the mineral (15). The :moval of matrix would then pro- action of a combination of col- ', 20) and lysosomal enzymes (21, experiments have demonstrated nzymes appear later than 24 hr ıe administration of parathyroid hat the delay is not due to a time l for enzyme activation after re- ;ested by the rapid action of col- rived from bone cells by Puzas (23). To date, no report has ap- ıe literature documenting the ef- thyroid hormone on the lipolytic bone. However, PTH has been duce lipolysis in rat epididymal *vitro* (24).

correlation of the appearance of ɔable of degrading phosphatidyl ı calcium release is, of course, ıstantial evidence that the two elated. The later appearance of ic activity parallels other reports ture and seems to indicate that :ntal conditions are, at least, anal- ogous to what has been observed *in vivo*. Previous reports have indicated that phosphatidyl choline, phosphatidyl ethanolamine, and sphingomyelin are the principal phospholipids extractable from bone matrix before demineralization (7). Following demineralization, phosphatidyl serine becomes extractable. While phospholipids have been implicated in bone formation at the epiphyseal line (7), there has been no role assigned to them in the diaphysis and indeed some data would actually suggest that they are not actively involved in bone formation in that area (25). Johnson (26) described histochemically a lipid layer on bone surface which is inactive, i.e., being neither formed nor resorbed. It appears that this layer is made up of cellular extensions, is permeable to large molecules, and probably plays an important role in calcium homeostasis (27). In conditions such as the hypophysectomized state (28) and following estrogen administration (29) there is an accumulation of lipids which might well be associated with an increase in the number of inactive surfaces. If this lipid layer is real, and all information would suggest that it is, it is reasonable to assume that removal or alteration of the lipid by appropriate enzymes would be a necessary step to calcium mobilization and subsequent bone resorption. This, then, is one possible explanation for the sequence of changes which has been described. Alternatively, the lipid removal could be associated with an increased turnover of some components of the surface cell membrane. It now seems probable that osseous tissue is separated from extracellular fluid by these cell membranes (30) and that the membranes have the ability to control ingress and egress of ions. It is not known whether there is a change in the cell membrane during the process but it is conceivable that the early mobilization of calcium which occurs after the administration of parathyroid hormone is associated with enzymatic alteration of the membrane.

Summary. The effect of 2 units of PTH on 10-day-old rat tibiae was studied. Incubations were carried out at timed periods up to 48 hr. The following parameters were measured: (a) the release of ^{45}Ca from prelabeled bone; (b) the release of [^{3}H]proline and [^{3}H]hydroxyproline from prelabeled collagen; (c) the re-

lease of [*methyl*-^{14}C]choline from prelabeled phosphatidyl choline. There was an early release of ^{45}Ca from bone fragments that paralleled the degradation of phosphatidyl choline. Collagenolytic activity was not demonstrable until later and reached its maximum 24 to 48 hr after the start of incubation. The data indicated that there is an increase in the breakdown of phospholipids which occurs at the time calcium mobilization is taking place. Degradation of at least a portion of the organic matrix of bone occurs later.

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ɔstaglandin D_2: Effects on the Rabbit Utero-Placental Circulation (40643)[1]

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ıle that prostaglandins play as regu- utero-placental blood flow has been ːly investigated in the past few years ıis work has concentrated on pros- ıs of the E and F series (1–3) and ently prostacyclin (4). A considera- on of prostaglandin metabolism *in* occur via the prostaglandin D path- .er than prostaglandin E or prosta- ⁼ (5). This has been demonstrated *in* ɔoth the rat brain and pregnant rat ', 7). There is little in the literature ʒ prostaglandin D's activity in the ɔn as it was reported a number of ɔ to be a biologically inactive pros- ı (5). More recent work in the cir- of the kidney and lung has demon- ıat prostaglandin D_2 (8) has similar to that of prostaglandins E or $F_{2\alpha}$ (9, have therefore extended the work ; the role of prostaglandins in the n of the utero-placental circulation ining the utero-placental and renal responses to prostaglandin D_2 in lly catheterized near-term rabbits.

ials and methods. Twelve New Zea- te rabbits weighing 2.8–4.8 kg were y prepared at 22 ± 2 days gestation. was performed with intravenous ıl (Abbott Laboratories) supple- with local Xylocaine anesthesia (As- ft ventricular polyvinyl catheter (i.d. ı) was placed via the carotid artery additional polyvinyl catheters were 7–10 cm in each femoral artery. Fem- eters were threaded subcutaneously eck incision and all catheters were ːh heparinized saline, 200 units/ml. sions were closed and the catheters ured under a bandage around the neck. Animals were allowed to recover for at least 24 hr prior to the experimental procedure. The arterial blood pressure of each rabbit was measured with Statham P23Db transducers and recorded with a Beckman R411 recorder. Heart rates were measured from the respective blood pressure tracings using a Beckman cardiotachometer coupler. Individual organ blood flows were determined using the radioactive microsphere technique. Fifteen-micron microspheres with either ^{125}I or ^{141}Ce label (3M Co.) were injected into the left ventricle while withdrawing an integrated arterial blood sample from the femoral catheter at a rate of 2.06 ml/min for 1.5 min. The spheres were suspended in 10% Dextran in saline. Each injection contained approximately 0.5×10^6 spheres at a volume of 0.15–0.20 ml. A stock solution of prostaglandin D_2 was prepared by dissolving 1.0 mg of prostaglandin D_2 in 1 ml of 99% ethanol which was stored at −20°. Appropriate dilutions were made with normal saline just prior to each infusion.

One microsphere injection of either ^{125}I or ^{141}Ce label was made just prior to the intraventricular infusion of prostaglandin D_2 (10 μg/kg/min). Immediately at the end of the 2-min prostaglandin D_2 infusion, another microsphere injection was made, using either ^{125}I- or ^{141}Ce-labeled spheres depending upon which isotope was used for the first microsphere injection. The maternal arterial pressure and heart rate were monitored continuously throughout the experiment. Following a 1-hr recovery period, an intraamniotic fluid catheter was placed, under Nembutal anesthesia, in 6 of the 12 rabbits. The effect of prostaglandin D_2 upon intrauterine pressure was determined by measuring amniotic fluid pressure during the infusion of prostaglandin D_2 (10 μg/kg/min) for 2 min.

Assay. Animals were sacrificed with euthanasia solution upon the completion of the

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experiment. The uterus and its contents as well as the kidneys were removed. The placenta for each fetus and its adjacent portion of myometrium were dissected free and each sample placed in a glass counting vial for assay. The kidneys were dissected into approximately 2-g sections and placed in glass counting vials. The integrated arterial blood samples and vials containing the tissue samples were counted on a three-channel Nuclear Chicago 1185 gamma counter and blood flows were calculated using a program on a Univac 1110 computer. Details of this technique have been described previously (11, 12). The resistances of the maternal organs were calculated by dividing the mean maternal arterial pressure (mm Hg) by the blood flow to that organ ((ml/g)/min). Resistance units are therefore expressed as mm Hg × min/ml × g. Results are expressed as the mean ± SE of the mean. Student's *t* test for paired data was used to compare the control and test observations.

Results. The blood flow data obtained in 12 rabbits in response to the prostaglandin D_2 infusion (10 μg/kg/min) are summarized in Table I. There was a small but significant reduction in mean arterial blood pressure from 87 ± 3 to 81 ± 3 mm Hg ($P < 0.01$). There was no change in the maternal heart rate. The uterine blood flow increased in response to prostaglandin D_2 from a mean of 0.14 ± 0.02 to 0.21 ± 0.03 ml/g/min ($P < 0.003$). Uterine vascular resistance decreased from 862 ± 164 to 544 ± 47 mm Hg × min/ml × g ($P < 0.01$). However, in the placenta, the blood flow decreased from 0.32 ± 0.03 to 0.16 ± 0.03 ml/g/min ($P < 0.001$) with a concomitant increase in placental vascular resistance from 389 ± 54 to 1136 ± 337 mm Hg × min/ml × g ($P < 0.03$). Prostaglandin D_2 dilated the renal vasculature. The renal blood flow increased from 4.23 ± 3.7 to 6.02 ± 0.88 ml/g/min ($P < 0.04$). Renal vascular resistance decreased from 23 ± 2.7 to 15.78 ± 1.5 mm Hg × min/ml × g ($P < 0.02$).

In the six rabbits in which amniotic fluid pressure was measured the prostaglandin D_2 infusion caused no change in the intrauterine pressures.

Discussion. The present study demonstrates that the rabbit uterine and placental and renal vasculatures are responsive to exogenously administered prostaglandin D_2. The formation of prostaglandin D_2 from arachidonic acid has been demonstrated in a variety of tissues including dog and rat renal tissue, rat brain, and gastrointestinal and uterine tissue (6, 7, 13, 14).

The circulatory regulatory capabilities of prostaglandin D_2 have only recently been examined. Jones compared the biological activity of prostaglandin D_2 in the sheep circulation to that of prostaglandin $F_{2\alpha}$ and prostaglandin E_2 (15). He found prostaglandin D_2 to have a weak depressor activity at higher doses versus a potent pressor activity at low doses. In addition prostaglandin D_2 exhibited a higher bioactivity than that of prostaglandin $F_{2\alpha}$. In this study we found prostaglandin D_2 to have a mild systemic depressor effect in the pregnant rabbit. No pressor effect was observed in preliminary experiments in which a lower dose of prostaglandin D_2 was utilized. This suggests a species difference in the pressor response which is supported by the observation that exogenous prostaglandin D_2 is a vasodepressor in dogs (16). The possibility also exists that there is modification in the pressor activity of prostaglandin D_2 during pregnancy. Jones (15) found pregnant ewes to be relatively insensitive to the pressor effects of exogenous prostaglandin D_2.

Individual organ responses to prostaglandin D_2 have been examined in the dog kidney and lung (9, 16). Prostaglandin D_2 increases lung airway resistance and increases pulmonary perfusion pressure in the dog, while strongly dilating the renal vasculature. In this study, prostaglandin D_2 dilated the rabbit renal vasculature.

TABLE I. THE UTERINE, COTYLEDONARY AND RENAL BLOOD FLOWS, AND VASCULAR RESISTANCES BEFORE (C) AND AFTER (T) A 2-MIN PROSTAGLANDIN D_2 INFUSION (10 μg/kg/min) TO 12 PREGNANT RABBITS

	Blood flow (ml/g/min)		Resistance (mm Hg × min/ml × g)	
Organ	C	T	C	T
Uterus	0.14 ±0.02	0.21 ±0.03	862 ±164	544 ±47
Cotyledons	0.32 ±0.03	0.16 ±0.03	389 ±54	1136 ±377
Kidney	4.32 ±3.7	6.02 ±0.88	23 ±2.7	15.78 ±1.5

has been considerable interest in the prostaglandins play in utero-placen- l flow regulation. Exogenous prosta- $F_{2\alpha}$ is a utero-placental vasoconstric- ıe pregnant sheep (3) while prosta- E_2 dilates this vasculature when ad- ɔd via the fetal compartment (3). En- s prostaglandins may be a partial ıant of the normal utero-placental ɔw in the pregnant monkey, rabbit, ɔp as prostaglandin synthetase inhi- sults in utero-placental vasoconstric- ll these species (2, 17, 18). In addition direct effect on the utero-placental ɔw, prostaglandins appear to be mod- ıf other vasoactive substances such as sin II and norepinephrine. This has monstrated both systemically in the t ewe (19) as well as in the utero- l circulation of the ewe and monkey and rabbit (2).

omplexity of the prostaglandin syn- ıthway lends itself to a modulatory ıe endproducts of arachidonic metab- ay shift depending upon the needs of n (8). In this sense it is important to the organ response to a variety of ındins. Prostaglandin D_2 is a potent l vasoconstrictor while mildly dilat- uterine vasculature. The divergence nse is similar to that noted by Novy) in the pregnant rhesus monkey. In experiments, prostaglandin E_2-in- terine contractions resulted in either ge or an increase in myometrial flow narked reduction in placental blood his pattern is identical to the one l in this study in response to prosta- D_2. Novy *et al.* did not discount the ty that vasomotor regulation of the rial arterioles might exist independ- he mechanical force of contraction. uction in myometrial resistance ob- n our study suggests that the vaso- hanges observed by Novy during la- r indeed be a chemically dependent ena especially since prostaglandin ıcrease markedly at parturition (22). r, the prostaglandin D_2 levels present abor are not known. In addition the t rabbit myometrium appears to be ve to endogenous vasoactive stimuli. ı severe enough to cause placental vasoconstriction has little effect on the myometrial vessels (23). The response to prostaglandin D_2 noted here may be due solely to a pharmacologic response with little correlation to a naturally occurring event.

Summary. The uterine, placental, and renal vascular responses to a 2 min intraventricular prostaglandin D_2 infusion (10 μg/kg/min) were examined using the microsphere technique in 12 chronically catheterized near-term rabbits. The prostaglandin D_2 infusion significantly decreased mean arterial pressure from 87 ± 3 to 81 ± 3 mm Hg. Uterine vascular resistance decreased from 862 ± 164 to 544 ± 47 mm Hg × min/ml × g while the placental vascular resistance increased from 389 ± 54 to 1136 ± 337 mm Hg × min/ml × g. The renal vasculature dilated in response to prostaglandin D_2, the renal vascular resistance decreasing from 23 ± 2.7 to 15.8 ± 1.5 mm Hg × min/ml × g. Thus, prostaglandin D_2 dilates the uterine and renal vasculature and is a strong placental vasoconstrictor. This response is independent of intrauterine pressure changes and appears to be a direct effect.

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Pituitary Enzyme Activities and Prolactin Secretion in Male Rats Treated with Psychotropic Drugs (40644)[1]

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ıl has been shown to influence hy- ɔ catecholamine turnover (1, 2) and e prolactin secretion directly (3, 4). ve reported that estrogen adminis- ɔ caused increased pituitary glyco- ıe activity which may be associated increased hormone synthesis (5). lies showed that estrogen feedback lactotrophic metabolic activity as ɔrmone production. Psychotropic ease prolactin secretion either by ɾpothalamic catecholamine metab- ɔy inhibiting their binding to the ic membrane receptors (3, 6). In t experiments we studied the effect ıtecholamine antagonists to manip- actin release on pituitary hexose- phate shunt, glycolytic, oxidative, ımal enzyme activities.

ls and methods. Mature, male, Dawley rats housed in a constant re room (22–23°) under artificial ɔn (0600–1800 hr) received 1.0 mg ine (Schering Company, Bloom-), 0.2 mg pimozide (Janssen Phar- ls, Beerse, Belgium), or 0.5 mg re- ɛrpasil, 2.5 mg/ml, CIBA Pharma- ompany, Summit, N.J.) s.c. daily . Twenty-four hours after the third the animals were decapitated. ɔod was collected for prolactin ra- oassay, and enzyme activities and ncentration in anterior pituitary tis- determined as previously reported ıetabolic pathways were character- e following enzymes: The hexose- ɔphate shunt by D-glucose-6-phos- ADP oxidoreductase, EC 1.1.1.49); glycolysis by ATP, pyruvate 2-*o*- ansferase, EC 2.7.1.40 (PK), and ate, NAD oxidoreductase, EC 1.1.1.27 (LDH); citric acid cycle by L-malate, NAD oxidoreductase, EC 1.1.1.37 (MDH), and by threo-D_s-isocitrate, NADP oxidoreductase, EC 1.1.1.42 (ICDH); and lysosomal breakdown by orthophosphoric monoester phosphohydrolase, EC 3.1.3.2 (tartrate-inhibited acid phosphatase).

Results. The injection of rats with psychotropic drugs produced serum prolactin levels which were increased 400% after perphenazine, 200% after pimozide, and 70% after reserpine treatment (Fig. 1).

Neuroleptic treatment did not change anterior pituitary weight significantly (Table I). The water-soluble protein concentration of the gland increased by 10–15%; the change was significant after perphenazine and pimozide treatment.

Enzyme activities (Tables II and III) are expressed as activity per milligram pituitary tissue, activity per milligram protein, and activity per pituitary gland. Perphenazine and reserpine did not affect G6P-DH activity, while pimozide increased its pituitary concentration and content slightly but significantly. Psychotropic drugs did not affect PK activity (mU/mg gland and mU/mg protein); after pimozide treatment the slightly enlarged glands contained more of this enzyme. LDH activity (mU/mg gland) elevated slightly only after perphenazine treatment. Perphenazine decreased ICDH activity (on all three bases) moderately; ICDH activity (mU/mg protein) also decreased after reserpine administration. MDH- and tartarate-inhibited acid phosphatase activities were not influenced by the treatments.

Discussion. It is well documented that catecholamines inhibit prolactin secretion and that their antagonists stimulate it. Increased synthesis of the hormone has also been reported following treatment with neuroleptics (3, 6). The present experiments showed a clear increase in serum prolactin concentra-

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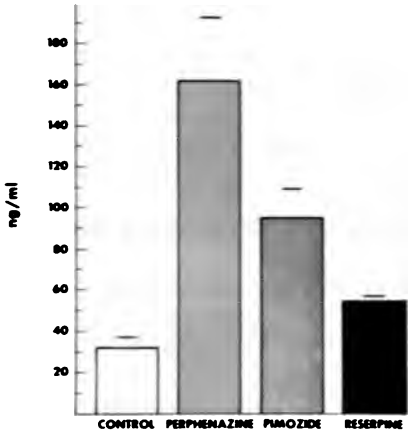

FIG. 1. Serum prolactin concentration of male rats treated with psychotropic drugs. The hormone concentration elevated significantly ($P < 0.01$) in each group after drug administration. All values are mean ± SEM. Injection schedule as in Table I.

TABLE I. EFFECT OF PSYCHOTROPIC DRUGS ON ANTERIOR PITUITARY WEIGHT AND PROTEIN CONTENT IN MALE RATS

Treatment	No. of animals	Anterior pituitary (mg)	Protein μg/mg gland	Protein μg/ gland
Control	8	7.63 ±0.26	91.3 ±5.9	699.0 ±51.1
Perphenazine	7	7.32 ±0.24	108.5[a] ± 4.3	791.6 ±33.9
Pimozide	8	8.39 ±0.31	104.3 ±4.9	872.7[a] ±46.9
Reserpine	7	7.06 ±0.31	108.1 ±5.3	754.6 ±24.8

[a] $P < 0.05$ versus controls. All values are mean ± SEM. Perphenazine 1.0 mg, pimozide 0.2 mg, and reserpine 0.5 mg were injected s.c. daily for 3 consecutive days. The animals were killed 24 hr after the last injection..

tions in male rats after perphenazine, pimozide, or reserpine treatment, while pituitary water-soluble protein concentration, oxidative enzyme activity, and tartrate-inhibited acid phosphatase activity remained unchanged.

Estradiol also increases prolactin secretion, primarily and directly stimulating synthesis of the hormone, which in turn causes increased prolactin release (3, 4, 8). In addition to its direct pituitary influence, estradiol inhibits dopamine release into the hypophyseal portal blood (1, 2), further stimulating release of the hormone. However, unlike the neuroleptics, which have no observable effect on lactotroph enzyme metabolism, estradiol causes increased glycolytic enzyme activity which appears to be associated with the estradiol-induced increases in prolactin synthesis (9).

The association of prolactin synthesis and enzyme activity has been observed in other systems as well. Rats bearing transplantable mammotropic pituitary tumors have atrophied pituitary glands with decreased prolactin synthesis (3). The administration of bromocriptine (3, 8) and of antiestrogens (10) to rats produces similar effects. In these cases, pituitary hexosemonophosphate pathway and anaerobic glycolytic activities also decreased significantly (7, 10). Numerous other morphological, physiological, and biochemical evidences support the hypothesis that total enzyme activities measured in pituitary homogenates reflect the metabolic activity of

TABLE II. ACTIVITY OF PITUITARY GLAND ENZYMES IN THE ANTERIOR PITUITARY OF MALE RATS TREATED WITH PSYCHOTROPIC DRUGS

Treatment	mU/mg gland	mU/mg protein	mU/ gland
Glucose-6-phosphate dehydrogenase			
Control	3.329 ±0.166	36.74 ±1.23	25.60 ±1.76
Perphenazine	3.654 ±0.128	33.78 ±0.78	26.72 ±1.29
Pimozide	4.066[a] ±0.199	38.92 ±1.52	33.99[b] ±1.78
Reserpine	3.666 ±0.242	33.76 ±1.18	25.64 ±1.66
Pyruvate kinase			
Control	18.98 ±2.78	200.2 ±17.6	145.0 ±21.4
Perphenazine	20.85 ±1.96	185.7 ±14.0	151.7 ±14.4
Pimozide	24.64 ±2.21	231.2 ±13.5	205.6[a] ±18.4
Reserpine	21.50 ±1.66	197.6 ±9.5	150.3 ±11.1
Lactate dehydrogenase			
Control	57.2 ±1.2	645.2 ±45.8	438.8 ±21.8
Perphenazine	62.1 ±1.3[a]	579.2 ±27.4	453.8 ±14.2
Pimozide	58.2 ±1.4	564.5 ±35.4	487.0 ±16.4
Reserpine	55.9 ±1.0	525.5 ±30.7	394.6 ±19.5

[a] $P < 0.05$ versus controls. All values are mean ± SEM. Injection schedule as in Table I.
[b] $P < 0.01$ versus controls.

TABLE III. ACTIVITY OF PITUITARY GLAND ENZYMES IN THE ANTERIOR PITUITARY OF MALE RATS TREATED WITH PSYCHOTROPIC-DRUGS

Treatment	mU/mg gland	mU/mg protein	mU/gland
Isocitrate dehydrogenase			
Control	2.200 ±0.123	25.28 ±2.86	16.90 ±1.23
Perphenazine	1.811[a] ±0.049	16.97[a] ±1.20	13.26[a] ±0.58
Pimozide	2.052 ±0.137	20.06 ±2.09	16.98 ±0.79
Reserpine	1.872 ±0.113	16.74[a] ±1.27	14.18 ±1.32
Malate dehydrogenase			
Control	277.5 ±7.5	3,148.4 ±260.2	2,130.3 ±114.0
Perphenazine	279.7 ±6.8	2,621.3 ±183.0	2,045.4 ±82.3
Pimozide	264.1 ±5.5	2,577.9 ±206.2	2,206.9 ±59.7
Reserpine	285.8 ±7.5	2,685.5 ±161.1	2,014.7 ±102.0
Tartarate-inhibited acid phosphatase			
Control	0.532 ±0.025	5.93 ±0.35	4.08 ±0.26
Perphenazine	0.558 ±0.021	5.20 ±0.29	4.06 ±0.11
Pimozide	0.508 ±0.033	4.91 ±0.37	4.23 ±0.25
Resperine	0.576 ±0.021	5.40 ±0.36	4.08 ±0.27

[a] $P < 0.05$ versus controls. All values are mean ± SEM. Injection schedule as in Table 1.

the lactotrophs (summarized in (5)).

We previously suggested that estradiol primarily stimulates prolactin biosynthesis, which increases promptly following administration of the drug (11). The demands of increased prolactin biosynthesis for newly synthesized glycolytic enzymes presumably activate the glycolytic metabolism of the lactotrophs (5). By contrast, the psychotropic drugs primarily stimulate prolactin release. Following their injection the gland releases previously synthesized hormone, greatly depleting its prolactin stores. The depletion may exert a positive intracellular feedback on prolactin biosynthesis (4, 12). New synthesis of mRNA or preprolactin have been observed (13), but subchronic psychotropic administration failed to stimulate the synthesis of newly formed glycolytic enzymes.

Hence these experiments suggest strongly that estrogen and neuroleptics stimulate prolactin synthesis through different mechanisms and that prolactin and enzyme synthesis have different gene loci in the lactotrophs, which can be stimulated by estrogen but not by the exclusion of the monoamines. While chronic administration of dopamine antagonists might stimulate the glycolytic metabolism of lactotrophs, that would represent a pharmacological situation far removed from physiological circumstances.

Summary. Psychotropic dopamine antagonists primarily stimulate prolactin release, and their subchronic administration does not influence the water-soluble protein concentration, the hexosemonophosphate shunt, and anaerobic glycolytic, oxidative, or lysosomal activity of the lactotrophs.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 194–198 (1979)

Modulation of Hapten-Specific Antibody Responses with Anticarrier Antibody: I. Differential Effects of IgM and IgG Anticarrier on Primary Direct and Indirect Hapten-Specific Plaque-Forming Cells (40645)[1]

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Antibody responses against a variety of antigens require the participation of T cells as helper cells. Such thymus-dependent antibody responses have been extensively studied in hapten-carrier systems. Evidence from a number of these studies suggest that T cells having carrier specificity are necessary for optimal hapten antibody responses. For example, passive transfer experiments demonstrate helper activity when carrier-primed T cells combined with hapten-primed B cells are transferred to irradiated donors receiving hapten-carrier conjugates (1). However, Kirov and Parish have recently shown that carrier-specific B cells participate in the helper effect (2). A 40 to 50% decrease in hapten-specific direct plaque-forming cells (PFC)[3] and 85 to 90% decrease in hapten-specific indirect PFC responses were observed after radioactive antigen suicide of the carrier-specific B cells. Thus, carrier-specific antibody may modulate the helper activity of T cells. Indeed, previous experiments have shown that passively administered antibody alters subsequent humoral responses to thymus-dependent antigens (3–8). Antibody-mediated modulation of thymus-dependent antibody responses has included both potentiation and suppression depending on the class or subclass of antibody used (3–5). For example, Henry and Jerne have shown that 19 S antisheep erythrocyte (SRBC) antibody potentiates whereas 7 S antibody suppresses the direct plaque-forming cell (PFC) response to SRBC (3). Some studies have shown a modulation of serum antihapten levels by passive administration of anticarrier antibody (5–8).

Since receptors for the Fc region of both IgG and IgM have been demonstrated on T cells (9, 10), it is conceivable these might bind to immunoglobulins complexed to carrier erythrocytes which are T-cell dependent. Such complex binding could thus initiate regulation of hapten antibody-producing responses through helper or suppressor T cells.

The following investigation evaluated *in vivo* fluctuations of a primary hapten antibody response induced by IgG and IgM antibodies specific for T-cell-dependent carrier erythrocytes. The hapten response was examined at the isolated single-cell level using Jerne's plaque-forming cell (PFC) assay enumerating modulations in both direct and indirect PFC (11).

Materials and methods. Homologous anti-bovine erythrocyte (ORBC) sera were prepared from the pooled sera of at least 20 mice 5 days after primary or tertiary immunization with 0.2 ml of 50% ORBC. The sera were fractionated by ammonium sulfate precipitation and Sepharose 6B column chromatography. The leading half of the 19 S peak from primary sera and the trailing half of the 7 S peak from hyperimmune sera were collected for use. Immunodiffusion with class-specific antisera revealed no cross-contamination of M and G and the latter contained both G_1 and G_2. Complement-dependent hemolytic titers of the fractions were determined using twofold dilutions of the antiserum fractions and an excess of guinea pig complement. To make comparisons between experiments using different pools of antisera, calculations

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[2] Recipient of a Research Career Development Award from the National Cancer Institute.

[3] Abbreviations used in this paper: PFC, plaque-forming cell; ORBC, bovine erythrocytes; TNP, 2,4,6-trinitrophenyl; SRBC, sheep erythrocytes.

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ıde using "hemolytic units." One he- unit is defined as the amount of y in 0.1 ml at the highest dilution ng complement-dependent hemolysis erythrocyte suspension.

o eight-week-old CBA mice of both :re used with 3 to 5 animals per group. mals were immunized i.p. with 0.2 ml TNP-ORBC immediately followed by :ction of the appropriate anticarrier y. One milliliter of ORBC was coated mg TNP for immunization (13, 14). /s 3, 5, 7, and 10, as indicated in the section, TNP-specific antibody-pro- cells from spleens were enumerated ıe Jerne plaque assay (11) with TNP- arget cells (13). Indirect plaques were ed with 1:400 dilution of rabbit anti- ;lobulin (Cappel Laboratories). Sheep :ytes alone were used as controls and ɔackground was subtracted from the RBC counts. The numbers of PFC are ıted as $\log_{10}$ mean ± SE per spleen. ɔund levels of TNP-specific PFC from ınized animals were 2.52 ± 0.08 (38) for direct PFC and 2.30 ± 0.05 (33) for indirect PFC. Log_{10} means have nverted to geometric means, as indi- the results section. Haptenic inhibi- direct and indirect PFC was accom- by adding varying concentrations of ɔupled to glycine (12) to the spleen they were mixed with the TNP-con- erythrocytes. Probabilities were de- d using a Student's *t* test.

ts. Kinetics experiments indicate both ct and indirect TNP-specific immune es peak in the mouse 5 days after n of TNP-ORBC conjugates (Fig. 1). y 5 responses in Fig. 1 represent the ic mean TNP-PFC/spleen from 28 and the other days represent geo- neans from 6 mice. The effects of IgM ɔ anti-ORBC on the hapten response ent times after injection are shown in Both classes of antibody potentiated nd indirect PFC on Day 3 after injec- ɔwever, at the peak of the response classes of anticarrier antibodies had ɔ effects on the TNP-PFC. Thus, on in this experiment IgM anti-ORBC ıted the direct response by >23,800 d potentiated the indirect response by

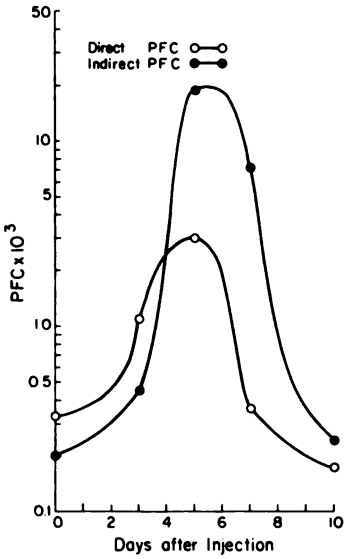


FIG. 1. Kinetics experiment of the primary PFC response specific for TNP. Spleens from CBA mice were assayed for PFC after being injected i.p. with 0.2 ml, 10% TNP-ORBC. Each point represents the geometric mean PFC/spleen from the following numbers of animals. Day zero represents data from 38 and 33 animals for direct and indirect PFC, respectively, Day 5 shows data from 28 mice for both direct and indirect PFC, and Days 3, 7, and 10 each from 6 animals each.

>110,000 PFC. In this experiment IgG anti-ORBC inhibited the indirect response by >43,000 PFC but had no significant effect on the direct response. Since the optimal TNP-PFC response was on Day 5 and because of the dramatic and opposite effects of the two classes of antibody on the antihapten response at this time, Day 5 was chosen for further analyses.

The results from multiple experiments in which animals were simultaneously injected with relatively high concentrations of IgM or IgG anticarrier antibody and antigen are summarized in Table II. IgM increased the geometric mean of the TNP-specific PFC by 5297 direct plaques and 47,448 indirect plaques. High concentrations of IgG mediated essentially no direct PFC suppression but reduced the mean number of indirect plaques by 16,671 PFC. Similar results have been obtained with several strains including CBA, C3H/HeJ, BALB/c, and (C56 B1/6 × DBA/2)F_1 mice and using either bovine or horse erythrocyte carriers and IgM or IgG

anticarrier antibodies. Haptenic inhibition studies in which varying concentrations of hapten coupled to glycine were added to the PFC overlay confirmed the TNP specificity of these PFC (data not shown here). Furthermore, the concentration of hapten (5 m*M*) required to inhibit 50% of the direct TNP-PFC was at least 10-fold greater than required to inhibit 50% of the indirect plaques indicating a higher average avidity of the indirect PFC. The IgM-potentiated direct and indirect PFC had the same low and high avidities, respectively, as the PFC from animals receiving antigen only.

TABLE I. *In Vivo* Kinetics of Modulation of TNP-PFC by Anticarrier Antibodies

Day after injection	Control[a]	Anti-ORBC IgM[b]	Anti-ORBC IgG[c]
	Direct PFC	Direct PFC	Direct PFC
3	2.73 ± 0.43[d] (573)	4.01 ± 0.12 (10,233)	3.78 ± 0.07 (6,026)
5	3.57 ± 0.10 (3,715)	4.44 ± 0.10 (27,542)	3.65 ± 0.07 (4,467)
7	2.37 ± 1.08 (234)	2.14 ± 0.60 (138)	3.25 ± 0.03 (1,778)
10	2.79 ± 0.26 (617)	3.27 ± 0.11 (1,862)	3.05 ± 0.09 (1,122)
	Indirect PFC	Indirect PFC	Indirect PFC
3	2.51 ± 0.55 (324)	3.83 ± 0.01 (6,760)	3.56 ± 0.32 (3,631)
5	4.55 ± 0.15 (45,709)	5.20 ± 0.05 (158,489)	3.53 ± 0.29 (3,388)
7	4.03 ± 0.07 (10,715)	NT[e]	3.24 ± 0.21 (1,738)
10	2.06 ± 0.27 (115)	3.12 ± 0.05 (1,318)	3.02 ± 0.33 (1,047)

[a] Control animals were injected with 0.2 ml, 10% TNP-ORBC.
[b] Animals received antigen and passive anticarrier IgM with 24 hemolytic units (defined in the text).
[c] Passive IgG anti-ORBC with 32 hemolytic units was injected with the antigen.
[d] Log_{10} mean ± SD of PFC/spleen from three animals tested separately. Numbers in parentheses represent the geometric mean.
[e] Not tested.

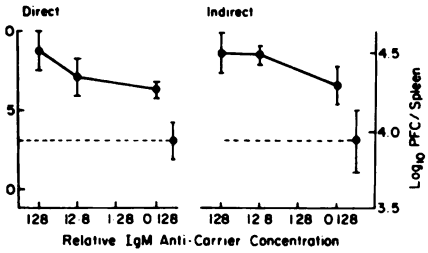
Dose–response experiments conducted on Day 5 with passive anticarrier IgM and IgG are shown in Figs. 2 and 3, respectively. Potentiation of both direct and indirect anti-hapten PFC was produced by IgM with carrier specificity. The maximum potentiation observed was three- to fourfold greater than controls receiving TNP-carrier conjugates alone (Fig. 2). The direct response was increased from 1940 PFC/spleen from animals receiving antigen alone to 7356 PFC by IgM anticarrier antibody. The number of indirect PFC was elevated from a control mean of 8481 to 30,528 plaques by passive IgM anti-ORBC. The potentiation in both direct and indirect PFC was dose responsive, i.e., decreased with decreasing concentrations of IgM.

As shown in Fig. 3, IgG anticarrier antibody had no significant effect on direct anti-hapten PFC. In contrast, higher concentrations of IgG anti-ORBC affected a 10-fold suppression of indirect TNP-specific PFC. It is conceivable that the hyperimmune sera used as a source of IgG might contain anti-

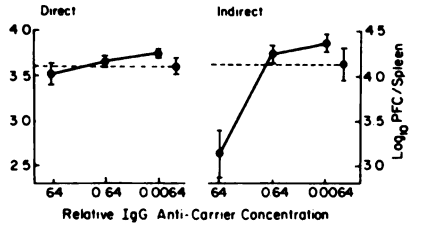
TABLE II. Anticarrier Antibody-Mediated Modulation of TNP-PFC Measured 5 Days After Injection

Group	PFC	No. experiments	No. mice	Log_{10} $\bar{x}$ ± SD	Geom $\bar{x}$	Δ PFC[a]	*P*
Control[b]							
Antigen only	Direct	9	28	3.48 ± 0.27[c]	3,020		
	Indirect	9	28	4.27 ± 0.33	18,621		
IgM[d]							
Anti-ORBC	Direct	5	14	3.92 ± 0.35	8,317	+5,297	<0.025
	Indirect	5	15	4.82 ± 0.26	66,069	+47,448	<0.0005
IgG[e]							
Anti-ORBC	Direct	9	31	3.42 ± 0.25	2,630	−390	NS[f]
	Indirect	9	31	3.29 ± 0.09	1,950	−16,671	<0.0005

[a] Calculated by subtracting the geometric means of the control PFC/spleen from the experimental means.
[b] Mice injected with 0.2 ml, 10% TNP-ORBC.
[c] Interexperimental variation.
[d] IgM hemolytic units (defined in the text) for each experiment ranged from 24 to 128, log_{10} $\bar{x}$ ± SD = 1.70 ± 0.28, geometric $\bar{x}$ = 50.
[e] IgG hemolytic units for each experiment ranged from 32 to 64, log_{10} $\bar{x}$ ± SD = 1.67 ± 0.11, geometric $\bar{x}$ = 47.
[f] Not significant.



2. Modulation of TNP-specific PFC by pas-
ıdministered anti-ORBC IgM. Spleens from at
ur CBA mice per group were assayed for PFC 5
ter being injected i.p. with 0.2 ml, 10% TNP-
and 0.1 ml anti-ORBC IgM (——). Control
from animals receiving only TNP-ORBC were
ayed (---). PFC were assayed with TNP-SRBC.
ly concentrations are represented in hemolytic
s defined in the text. Maximal potentiation of
ecific direct and indirect PFC was significant at
05 and $P < 0.025$ levels, respectively.



3. Modulation of TNP-specific PFC by pas-
ıdministered anti-ORBC IgG. Spleens from at
ur CBA mice per group were assayed for PFC 5
ter being injected i.p. with 0.2 ml, 10% TNP-
and 0.1 ml anti-ORBC IgG (——). Control
were assayed from animals receiving only TNP-
(---). PFC were assayed with TNP-SRBC. An-
oncentrations are represented in hemolytic units
ed in the text. Significant ($P < 0.0125$) suppres-
indirect hapten-specific PFC was observed with
est concentrations of IgG anti-ORBC.

pic antibody against carrier-specific re-
s. Thus, idiotype suppression (15) could
ıechanism involved in the IgG-induced
ssion of hapten-specific PFC. We,
ore, evaluated whether anti-ORBC IgG
l to the carrier would produce this sup-
on. Injection of TNP-ORBC conju-
pretreated with IgG anticarrier and
washed, resulted in comparable sup-
on of the indirect PFC against TNP
not shown). Thus, IgG with carrier
icity alone is sufficient to produce this
ssion.

cussion. The data presented here show
assive antibody specific for a T-depend-
ent carrier can modulate the *in vivo* primary PFC response to a defined haptenic determinant. An increase in the numbers of both direct and indirect TNP-specific antibody-producing cells in mouse spleens was produced by both classes of anticarrier antibodies 3 days after injection. However, at the peak response, 5 days after injection, IgM continued to produce potentiation of both direct and indirect PFC. Anticarrier IgG had little effect on the 5-day direct hapten-specific PFC. However, the indirect hapten responses at 5 days were consistently suppressed by anticarrier IgG at relatively high antibody concentrations.

One possible explanation for these data is that IgM anticarrier activates helper T cells, potentiating both the direct and indirect PFC responses to hapten; and IgG, in relatively high concentrations, activates suppressor cells which either inhibit helper activity or directly suppress the indirect PFC response to hapten. Inhibition of indirect PFC could also be a result of IgG anticarrier antibody covering sites necessary for helper-cell recognition. Inhibition by simple blocking of antigenic determinants seems unlikely, however, since $F(ab)_2$ fragments of anticarrier IgG have been shown to be much less efficient than intact IgG in antibody-mediated suppression (4, 16). The structures necessary to interact with antibody to produce help or suppression of a thymus-dependent response would be receptors on T cells for IgM Fc (10) and IgG Fc (9), respectively. In support of such a model (although their system was not antigen specific), Moretta *et al.*, using human lymphocytes, have recently shown that T cells bearing IgM Fc receptors can function as helper cells and T cells bearing IgG Fc receptors can function as suppressor cells (17). Furthermore, T cells have been found essential in antibody-mediated potentiation of immune responses to erythrocytes (18) as well as in a hapten-carrier system where the hapten response was potentiated by anticarrier antibody (19). Also, as indicated above, the Fc region of IgG has been shown to be important in IgG-mediated suppression of antibody responses (4, 16).

Since T cells have receptors for the Fc regions of immunoglobulins, it is reasonable to assume immune complex binding to lymphocytes has a functional role that may well

be of a regulatory nature. The present study suggests activation of helper or suppressor T cells by IgM and IgG anticarrier antibodies, respectively, which bind to the carrier through their antigen combining sites and to the T cells via IgM or IgG Fc receptors.

The experiments presented here provide no direct evidence for the involvement of T cells in this antibody-induced potentiation or suppression. However, evidence from a number of studies make it seem probable that T cells are involved: 1. The responses studied are thymus dependent; 2. it has been demonstrated that antibody-mediated modulation of such responses requires T cells (18, 19); 3. finally, the demonstration of immune complex receptors on T cells (9, 10) would predict some functional significance for these structures. Ultimate resolution of this problem will require passive transfer experiments with purified populations of cells.

Conceivably, the observed modulations in the hapten-specific responses could involve an indirect effect on helper or suppressor cells via amplifier T cells (20). Alternatively, antigen–antibody complexes could activate macrophages to modulate the hapten-specific responses (21).

Whatever the mechanism involved, the magnitudes of the observed *in vivo* modulations are very large and statistically highly significant. As much as 10-fold suppression of indirect PFC was produced by IgG and 4-fold potentiation of both direct and indirect PFC was produced by IgM. These anticarrier antibody-mediated modulations may represent *in vivo* immunoregulatory phenomena since anticarrier IgM and IgG are components of the host primary immune response.

Finally, these findings may offer promise in terms of pragmatic manipulation of the immune response; helper activity against undesirable antigens might be potentiated by attaching IgM to another (carrier) portion of the molecule. Specific suppression also may be activated using IgG anticarrier antibodies.

Summary. Immunization of mice with TNP coupled to ORBC resulted in a TNP-specific antibody response. TNP-specific direct and indirect PFC in the spleens reached a maximum level 5 days after injection. Passively administered anticarrier antibodies injected with the hapten-carrier conjugates were found to modulate the primary PFC response to hapten. Three days after injection, both classes of carrier-specific antibody induced potentiation of hapten-specific direct and indirect PFC. On Day 5, however, IgM and IgG had opposite effects on the TNP-specific response. Carrier-specific IgM produced a three- to fourfold increase in both direct and indirect hapten-specific PFC over animals injected with the hapten-carrier conjugates alone. In contrast, carrier-specific IgG, at relatively high concentrations, suppressed the hapten-specific indirect PFC but had little effect on direct hapten-specific PFC.

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OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 199-201 (1979)

Comprehensive Profile of Peripheral Progesterone Levels in Cyclic Hamsters Sequentially Bled at 2-hr Intervals (40646)

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irst assessments of the peripheral pro- ıe (P) profiles during the 4-day estrous the hamster were done nearly a dec- (1, 2). The experimental design used and virtually all subsequent studies ıer to make a single determination of n the blood at each of the first 3 days ycle and multiple determinations on of proestrous, Day 4 (1–4), or to : in detail a restricted period (24 hr or the cycle (5–7). The resultant limited from these studies were relatively even though (a) the assay methodol- ied from gas–liquid chromatography competitive protein binding (2, 5) to munoassay (4, 6–8), and (b) the ›f the analyzed peripheral blood var- ı abdominal aorta (2, 5), vena cava mixed trunk blood (4, 6, 7) to ovarian blood (3). In contrast to the experi- design of those studies, a design used ey and Greenwald (8) incorporated 'e cardiac puncture of hamsters in ınd yielded a detailed profile of pro- ıe levels in the blood at 2-hr intervals out the estrous cycle. Their resultant ›f P indicated that a series of episodic ions in P levels existed during the ıalf of Day 4 (proestrus) and contin- ›ugh Day 1 of the next cycle. The ınt fluctuations in P on Day 4 had not ınd in the previous (1, 2, 5) or sub- (4, 7) studies. In view of these collec- orts, the present study was done to comprehensive profile of P levels in d by using a more direct experimental The rationale for the study was that ›dic fluctuations in P found in Ridley eenwald's study could have been a ence of "animal variation" (8), which ce was manifested through their par- xperimental design.

ials and methods. Female golden s (Con Olson, Madison, Wis.), weighing 90–110 g, were maintained in an environmentally controlled room (22°, lighting from 0500 to 1900 hr daily, CST). The estrous cycle was monitored by the appearance of a characteristic vaginal discharge every fourth day, which was designated Day 1 of the cycle (the day of ovulation). After five consecutive 4-day cycles, eight hamsters (two at each day of the cycle) were concurrently bled (0.08 ml, 27-gauge needles) via cardiac puncture without anesthesia at 2-hr intervals for a 24-hr period (starting at 0800 hr). Bleedings were done in the animal room except during the period of darkness, when animals were rapidly but quietly removed to a very dimly lighted anteroom for at most a 10-min period. After the 24-hr period for blood collection, hamsters were monitored again for subsequent vaginal discharge. The experiment was replicated three times. Animals which failed to show two subsequent and normally timed discharges were excluded from the study. The collected blood was allowed to clot at 4° for 12–16 hr, centrifuged at 1200*g*, 4° for 0.5 hr, and then 10 μl sera was diluted with 0.5 ml distilled water and stored at −15°.

For determination of P, prediluted sera, nonradioactive P for reference standards, and control reference sera (with and without unlabeled P, or with labeled P for analysis of procedural loss through the extraction process) were extracted once with 3 ml anhydrous diethyl ether, and phase separation was facilitated by centrifugation at 500*g*, 22° for 40 sec. The aqueous phase was frozen (dry ice, ethanol bath); the ether phase was decanted into a 12 × 75-mm culture tube and then evaporated under air in a 45° water bath. The etheric extract was sequentially treated as follows: twice concentrated and dried via ether washes (0.5 and 0.25 ml); resuspended in 0.2-ml phosphate-buffered gelatine (PBG; 0.1 *M* phosphate, pH 7.0, containing a 0.14 *M* sodium chloride, 0.1% Knox gelatin, and

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0.1% sodium azide); gently agitated, and incubated at 45° for 20 min and then 22° for 10 min. To each culture tube was added 0.1 ml anti-P serum (1:62,000 dilution in PBG, antiserum AB12 kindly supplied and characterized by Dr. A. H. Surve and associates (9)), and then 0.1 ml [1, 2, 6, 7-^{3}H]labeled-P (equivalent of 19 pg P added; original SA = 105 Ci/mmole) in PBG. Tubes were agitated and then incubated at 22° for 16–20 hr and at 4° for 1 hr. Free and bound P were separated at 4° by the following sequence: addition of 0.5 ml dextran-coated charcoal suspension (0.4% Norit-A and 0.04% dextran in PBG) to each tube; contents agitated; incubation (15 min); (1500*g*, 15 min); decantation of supernatant into 6-ml scintillation vials and subsequent addition of 5 ml scintillation solution [15 g Omnifluor (New England Nuclear, Boston)/gallon of 10% dioxane in toluene]. After a 20- to 24-hr incubation, vials were counted for 10 min or 10^4 accumulated counts in a Packard Tri-Carb liquid scintillation counter, Model 3003. P content was calculated from the transformed standard curves (log-dose vs logit-response) after linear regression analysis and included correction for procedural loss. Cumulative characteristics for 19 assays were as follows: (a) lower limit of sensitivity = 5 pg; (b) mean (±SE) slope and the intercept at 50% binding were −2.465 ± 0.038 and 72.5 ± 1.8 pg, respectively; (c) percentage recovery of labeled internal standard after ether extraction was 83.8 ± 0.5% (n = 18); and (d) net recovery of nonradioactive P when 100 pg P was added to reference sera was 101 ± 3 pg. Levels of P in sera were analyzed by two-factor analysis of variance for repeated measure on one factor followed by Duncan's new multiple-range test (10), with a level of probability at less than 0.05 considered statistically significant.

Results and discussion. Three hamsters were excluded from the study (two at Day 2, one at Day 3) because they failed to exhibit a vaginal discharge at the expected, *second* postbleeding Day 1. The remaining 21 females appeared healthy throughout the study and exhibited two normally timed vaginal discharges after the bleedings.

The results are presented in Fig. 1. Overall P levels were similar in hamsters bled over a 24-hr period initiated on Days 1 or 2 but were significantly greater in Day-1 or -2 hamsters than the P levels in Day-3 animals. Also, the levels of P in animals in which serial bleeding was initiated at 0800 hr on Day 4 were significantly elevated compared to 24-hr levels in the groups of hamsters bled at the other days of the cycle. A noteworthy point is that both the levels of P and the pattern of peripheral P levels found between 1000 hr, Day 2

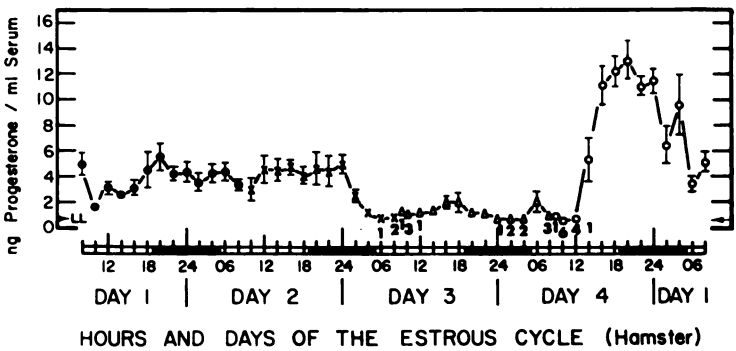

FIG. 1. Peripheral levels of progesterone in the cyclic hamster. Animals in each of four groups were bled (0.08 ml/bleeding) by cardiac puncture without anesthesia at 2-hr intervals over a 24-hr period beginning at 0800 hr on Day 1 ((●); n = 6), Day 2 ((×); n = 4), Day 3 ((△); n = 5), or Day 4 ((○); n = 6), respectively. Each symbol represents the mean (±SE) progesterone concentration; at some hours the symbol for the mean overlapped the limit of the SE. LL on the ordinate indicates the lower limit of detection of progesterone, in 10 μl of serum, on a concentration basis. The numbers beneath the symbols at Days 3 and 4 indicate the number of hamsters that had nondetectable levels of progesterone at the particular hours (these animals were not included in the calculation of the mean progesterone level depicted by the symbol at the corresponding hour). The dark bars along the abscissa represent the darkness periods of the lighting cycle.

00 hr, Day 3 in the present study using diac puncture technique are virtually al to the levels and pattern recently ined in sera from decapitated hamsters particular time frame (6).

ntrast to patterns of P levels reported ley and Greenwald (8), no significant c fluctuations over successive 4-hr pe- ere found on either Days 4 or 1 in the study, when hamsters in a *single group* erially bled at 2-hr intervals during 24-hr period. In the former study (8), ltiple peak levels of P on Days 4 and virtually from two groups of animals bled at 4-hr intervals over consecutive riods (2000, 2400, 0400 hr, and 0600, 400 hr, respectively) while the recip- w levels of P on these days were from her groups of animals serially bled in sign (1800, 2200, 0200 hr, and 0800, 600 hr, respectively). Comparisons of signs and results of these two studies sults of other studies, which assessed P rimarily on Day 4 (1, 3, 5, 7) and with the present results agree, suggest that ies of episodic fluctuations found by and Greenwald were most probably chance variation in tonic peripheral P between aforementioned groups of rs and were manifested by their ex- ntal design. The same explanation ap- qually plausible for the significant el- s found on Day 3 (8), which contrast vels in the present study and in sera decapitated hamsters (Bast, unpub- . Although only the composite profiles en in Fig. 1, the 24-hr profile of indi- animals at various stages of the cycle ed an occasional sporadic elevation, however, did not consistently occur in nsters at particular hours during the hus, the results of the present study e that the peripheral P levels are rela- table within each day of the cycle, at hen determined at 2-hr intervals in bled animals, except during deline- ansitional periods of altered ovarian n (e.g., the preovulatory rise in P after 1200 of Day 4; during luteinization on the first half of Day 1; and during a period of rapid luteolysis early on Day 3).

Summary. A composite profile was made of peripheral progesterone (P) levels at 2-hr intervals over the 4-day estrous cycle of the hamster. Specifically, each of four groups of animals was bled via cardiac puncture (0.08 ml blood/bleeding), without anesthesia, at 2-hr intervals for a 24-hr period starting at 0800 hr on different days of the cycle. P was measured in etheric extracts of serum by RIA. After a fairly steady threefold increase in P from 1000 to 1800 hr on Day 1 (day of ovulation and vaginal discharge), P levels remained relatively stable at 4–6 ng/ml until 2400 hr on Day 2. During the first 6 hr on Day 3, P levels declined to near nondetectable values (<1 ng/ml) and thereafter fluctuated little until the dramatic preovulatory rise began between 1200 and 1400 hr on Day 4 (to 11–13 ng/ml by 1600 hr). After 2400 hr on Day 4, levels of P declined to 4–6 ng/ml by 0600 hr on Day 1. Series of close-interval, episodic fluctuations in P levels were not found.

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Persistence and Expression of Herpes Virus in Guinea Pig B and T Spleen Cells (40647)

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A herpes-like virus (GPHLV) was originally isolated from kidney cell cultures derived from leukemic strain 2 guinea pigs (1). Subsequently, the virus was also isolated from leukocytes and other tissues of nonleukemic strain 2, strain 13, and occasionally Hartley guinea pigs without clinical disease (2). Although virus particles or inclusions could not be found in tissues obtained from either naturally or experimentally infected guinea pigs, the virus could be isolated following cultivation or cocultivation of the infected cells. Earlier reports have shown that the virus is widely distributed in leukocytes and various tissues; however, the highest virus titers were always demonstrated in the spleen (2–5). The detection of GPHLV particles in guinea pig leukocytes only after *in vitro* cultivation resembles the reported observations of Epstein–Barr virus (EBV) particles in cultured human leukocytes or tumor cells (6). The main target for EBV has been defined as the human B lymphocytes (7) and it has been shown that normal human lymphocytes, which have a limited life span *in vitro*, will persist in culture if the EBV genome is present in the cells (8, 9). The ability of GPHLV to convert normal lymphocytes into permanently growing cell lines has not been demonstrated, although a limited transformation of circulating guinea pig leukocytes has been described after *in vitro* infection with GPHLV (10). The specific cell type in which GPHLV persists has not been determined; GPHLV particles have been seen in bone marrow macrophages after *in vitro* cultivation, but it was not certain whether they were part of the cell genome or only the result of the macrophage ingesting the virus secondarily (11). Identification of the specific cell type harboring the GPHLV genome is of particular interest since guinea pig leukemia has been classified as a B-cell proliferation (12).

Materials and methods. Virus strain. The LK40 strain of GPHLV was originally isolated from a leukemic guinea pig and was used throughout this study. The virus was serially passaged in guinea pig embryo cell monolayer cultures. Virus infectivity titers of the stock virus varied from 10^6 to 10^7 $TCID_{50}$/ml.

Tissue culture for virus assay. Guinea pig embryo fibroblast (GPE) monolayer cell cultures were prepared from 30- to 40-day-old fetuses of normal Hartley guinea pigs. They were grown in Eagle's basal medium with 10% fetal bovine serum as described previously (13). GPHLV infectivity titers were measured by inoculating serial 10-fold dilutions of infected tissue culture suspensions into GPE monolayer cultures. Virus infectivity titers in separated spleen cells were evaluated by cocultivation of serial 10-fold dilutions of 10^6 cells with GPE monolayer cell cultures.

Animal inoculation. Virus-free Hartley guinea pigs were inoculated intraperitoneally with 5 ml of 10^6 to 10^7 $TCID_{50}$ per milliliter of GPHLV. Animals were sacrificed at different intervals after inoculation and the spleens were removed, minced, and separated into the different subpopulations as described below.

Spleen cell separation. Spleens were removed aseptically from uninfected or infected guinea pigs. The spleens were minced and the cell suspension obtained was washed, resuspended in RPMI 1640 medium with 20% fetal bovine serum, and separated into cell fractions according to the following procedure. The red blood cells were lysed with a solution of ammonium chloride (0.83%). The spleen cells were allowed to adhere to a glass surface for 1 hr at 37° and the glass-adherent

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action was removed by scraping the urface. The cell population which did lhere to the glass was then separated ractions enriched in B or T cells on wool columns, as described by Julius with some modifications (14). Before ation of cells, the columns were washed Hanks' balanced salt solution (HBSS), orated with RPMI 1640 medium for 1 37°, and then rinsed with fresh RPMI nedium. About 3 ml of cell suspension ning 10^7 to 10^8 cells/ml were applied to umn. The cells that passed directly gh were reconcentrated by centrifuga- id approximately 10^7 cells were applied cond column which was then incubated for 1 hr. The enriched B-cell fraction ent to the first column was obtained by us elution of the cells from the nylon The cells that did not adhere to the l column were considered the T-cell- ed fraction. All enriched cell fractions pelleted, resuspended in fresh RPMI nedium with 20% fetal calf serum, and mber of viable cells was determined by i blue exclusion.

characterization. The T cells were enu- ed by rosetting with papain-treated rab- rthrocytes as described previously (15, cells were characterized by a direct e fluorescence assay using rabbit anti- i pig immunoglobulin (17). Macro- s were identified by their capacity to e to glass and to phagocytize latex par-

tivation of lymphocytes. Aliquots of B- -enriched fractions containing 3 to 5 × lls/ml either obtained from uninfected ected animals were kept in culture in 1640 medium with 20% fetal bovine . The cultures were supplemented y with fresh media. Lymphocytes in- *in vitro* with virus were kept in the manner. Viable cells were enumerated nd identified at selected time intervals to 4 weeks; virus infectivity titers were ined in parallel.

ults. Localization of GPHLV in sepa- spleen cells derived from acutely and y infected guinea pigs. Enriched frac- obtained from acutely or latently in- guinea pigs were cocultivated with fibroblasts on the day of sacrifice, and GPHLV virus infectivity titers per 10^6 cells were determined. The results obtained during acute GPHLV infection, 1 to 18 days, and latent infection, 25 to 60 days after virus inoculation, are shown in Table I. GPHLV was detected in the macrophage and B-cell fractions as early as 1 day after inoculation whereas the virus was not detected in the T-cell fraction during the first 4 days postinoculation. GPHLV was only detected in the T fraction in animals sacrificed 5 days postinoculation or thereafter. Virus infectivity titers showed an increase of approximately one log in all three cell fractions from day 1 to 18. GPHLV infectivity titers of spleen cell populations obtained from latently infected guinea pigs indicated that GPHLV was isolated from all three fractions. However, the highest virus titers were consistently measured in the B-cell fraction, and the lowest titers were always evident among the T cells.

Expression of GPHLV after in vitro cultivation of B- and T-cell fractions derived from infected guinea pigs. In order to assess GPHLV expression, B and T cells obtained from latently infected guinea pigs were cultured *in vitro* for 20 days and virus infectivity titers and cell count were determined daily. The results are shown in Fig. 1. On Day 0, cell counts were high but virus titers were low since the virus was not as yet expressed. Cell counts in each population decreased slowly thereafter but virus titers increased gradually due to expression of GPHLV in both B- and T-cell fractions. On the basis of four experiments, maximum virus expression was noted in the T-cell fraction after 8 days in culture, whereas 13 days were necessary to obtain

TABLE I. LOCALIZATION OF GPHLV IN SEPARATED SPLEEN CELLS OBTAINED FROM GUINEA PIGS DURING ACUTE AND LATENT INFECTION[a]

Days postinoculation	No. Expts	Average $\log_{10}TCID_{50}/10^6$ cells: Whole spleen	[M]	[B]	[T]
1–2	5	0.28	0.23	0.24	0[b]
3–4	3	0.54	0.88	0.31	0
5–8	5	0.97	1.19	1.11	0.36
11–18	4	1.71	1.58	1.62	0.78
25–60	10	1.88	2.21	2.56	1.47

[a] Each animal was inoculated with 10^7 $TCID_{50}$ intraperitoneally. [ ], enriched population; M, macrophages; B, B lymphocytes; T, T lymphocytes.

[b] Virus was not detected.

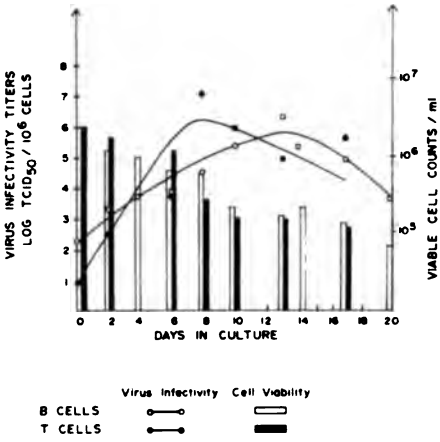

FIG. 1. Expression of GPHLV after *in vitro* cultivation of spleen cell fractions derived from latently infected guinea pigs; average of four experiments. Each animal was inoculated with 10^7 $TCID_{50}$ and sacrificed 25 to 60 days postinoculation.

maximum virus expression for the B cells.

In vitro cultivation of B and T spleen cells with or without GPHLV. In order to determine if spleen cells could support the replication of GPHLV *in vitro*, spleens were obtained from noninfected guinea pigs and were separated into subpopulations. Cell identification in each of the subpopulations on the day of sacrifice showed that about 90% of the cells in the T fraction formed rosettes, approximately 85% of the cells in the B population presented surface fluorescence, and about 90% of the cells in the macrophage fraction phagocytized latex particles. B- and T-cell-enriched fractions were then infected with GPHLV (MOI of 1) for 1 hr at 37°. Unadsorbed virus was removed by three successive washings with HBSS. The infected cells were resuspended in RPMI medium with 20% fetal bovine serum at a concentration of 3 to 5 × 10^6 cells per ml and incubated at 37°. Cell counts and virus infectivity titers were determined daily for 18 days (Fig. 2). GPHLV infectivity titers decreased rapidly in the absence of cells. Although B- and T-cell fractions infected *in vitro* with GPHLV showed no significant increase in virus titer during the 18 days of cultivation, virus infectivity titers decreased at a much slower rate than those without cells. It was also noted that the number of viable cells decreased in both B- and T-cell fractions, although the survival rate of the noninfected cells was better than that of infected cells (Fig. 2). In addition, a diminution in the number of rosette-forming cells in the T fraction and in the number of fluorescent cells in the B fraction was observed after 6 to 12 days in culture. Neither virus titers nor cell viability was increased when similar experiments were conducted with spleen cells that had been kept in culture for 2 or 6 days before virus inoculation.

Discussion. The role of bone marrow-adherent cells in the latency of GPHLV was explored in a previous study by our laboratory (11). Data indicated that GPHLV was present *in vivo* in the macrophage-enriched bone marrow fractions of latently infected guinea pigs. The present study showed that the different spleen subpopulations obtained from latently infected animals also harbored the virus. GPHLV was not only isolated from the macrophage-enriched fraction but also from the B- and T-cell-enriched spleen pop-

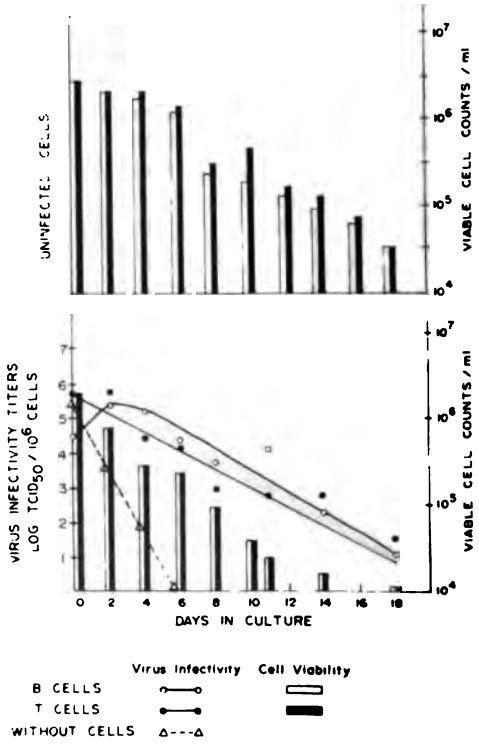

FIG. 2. Cultivation of B and T cells *in vitro* with or without GPHLV infection (average of four experiments). Virus inoculum on Day 0 was 10^6 $TCID_{50}/10^6$ cells.

ıs; however, the highest GPHLV infec-
iters were consistently measured in the
fraction. Results obtained in acutely
d animals demonstrated that GPHLV
be detected in B cells as early as 1 day
noculation whereas isolation of virus
the T-cell subpopulation was not
ed until 5 days or more after virus
ation. Furthermore, the lowest infectiv-
rs were always observed in the T frac-
uring latent infection. Since each en-
fraction contained an average of 10%
er cells, it is possible that the presence
ontaminating cell harboring GPHLV
ıts in part for the presence of the infec-
virus detected in each cell fraction.
/er, despite the technical restrictions
ed as a result of contamination by other
ɔes, it appeared that in latently infected
. pigs, GPHLV localized in the B cells
ntially. The presence of GPHLV in T
ɔuld not be ruled out since this fraction
nly rarely been found free of virus.
r, whether the virus observed in the
macrophages was an integral part of
l genome or whether it occurred as a
of phagocytosis of an infected cell re-
to be determined.

HLV expression in organ cultures of
s and spleen tissues and in adherent
marrow cells derived from infected
. pigs has been described (4, 11). The
t study showed that GPHLV was also
sed in cultured B and T lymphocytes
d from infected guinea pigs. It ap-
l that B cells, T cells, or an additional
ction might be responsible individually
ssociation for viral expression *in vitro*.
l, the enriched B- and T-cell popula-
hat were infected with GPHLV con-
approximately 10% of contaminating
n addition, the number of rosette-form-
ls in the T fraction and in the number
rescent cells in the B-cell fraction de-
d after several days in culture. It was
rtain whether this was due to the loss
specific cell receptors or to the prefer-
growth of non-T, non-B cells during
tion.

ontrast with what has been observed
one marrow macrophages (11) neither
T spleen cells were able to support
V replication when infected *in vitro*.
Serial counts of viable cells showed that the number of B and T cells infected *in vitro* declined at a faster rate than both uninfected controls and cells infected *in vivo*. This was probably due to the lytic effect of GPHLV on guinea pig leukocytes. It is possible that the use of lower MOI and/or mitogen-activated lymphocytes might be more satisfactory for the *in vitro* infection of lymphocytes with GPHLV.

Natural infection of leukocytes has been described for many herpes viruses in both humans and animals. In addition, a number of herpes viruses have been shown to be capable of infecting and replicating in lymphocytes (6, 18–21). Several reports have indicated that some herpes viruses are closely associated with T lymphocytes (20, 21) whereas others, including EBV, have been classified as B lymphotropic (7). We report here that B cells, T cells, and macrophages obtained from the spleen of GPHLV latently and acutely infected guinea pigs harbor GPHLV, although there is some evidence of a GPHLV tropism for B cells. The capacity of GPHLV to persist in lymphocytes and travel to various tissues as well as the possibility of affecting the immunocompetence of such cells may be significant for understanding the pathogenesis of GPHLV as well as other latent herpes virus infections.

Summary. The role of spleen lymphocytes in acute and latent guinea pig herpes-like virus (GPHLV) infection and the *in vitro* susceptibility of the lymphocytes to GPHLV were explored. Macrophage-, B-cell-, and T-cell-enriched populations obtained from infected guinea pigs were examined for infectious GPHLV. During acute infection, virus was first detected in the macrophage and B-cell fractions, whereas, infectious virus was only evident in the T-cell fraction 5 days or more after inoculation. During latent infection, infectivity titers in the B fractions were consistently higher than in the T fractions. In both the B and the T lymphocytes derived from latently infected guinea pigs, virus was expressed only after *in vitro* cultivation or cocultivation with susceptible cells. Lymphocytes infected *in vitro* did not support GPHLV replication, although latent infection of lymphocytes with GPHLV was readily accomplished *in vivo*.

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Isoproterenol-Induced Hypokalemia: Role of the Liver (40648)[1]

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1933, D'Silva and Bachromejew, work-
ıdependently, reported the influence of
mpathetic nervous system on the potas-
concentration, [K], of the blood (1, 2).
va found that intravenous injection of
phrine in the cat caused arterial serum
) increase temporarily, then to decrease
ly below the control value, and even-
to return to normal (3).
Silva (4) and others (5–8) presented ev-
ɛ that the liver is the major source of the
ıse of arterial [K] caused by epineph-
Other work indicates that the hypoka-
, caused subsequently by epinephrine,
: to the uptake of K by both skeletal
e (8–12) and the liver (8, 9, 13). Kastello
'ick (13) found that the gut first took up
ring the hyperkalemia, caused by epi-
ine, and then released K during the
ɛalemic phase.
temic infusion of the adrenergic β-re-
r agonist, isoproterenol, causes only ar-
hypokalemia (14, 15). Isoproterenol has
shown to cause an uptake of K by
al muscle during local infusion, and this
e is not related to any change of blood
(11, 12); investigators have postulated
3-receptor agonism alone may lead to
)f K by the liver (8). Castro-Tavares and
)so (16) stated that an isolated dog-liver
ration seems to have β-receptors, which,
stimulated, cause the uptake of K.
, one of the same investigators reported
ıeither systemic infusion of isoprotere-
or injection of the drug into the hepatic
', affected liver K (17).
have studied the role of hepatic tissue
production of hypokalemia by isopro-
)l, utilizing administration of the compound by constant infusion into the portal vein in the dog. Our results establish that the liver takes up K when adrenergic β-receptors are stimulated, and that the liver need not lose K for the uptake to occur.

Methods. Dogs that weighed between 12.5 and 29.5 kg and were not selected for sex or breed were anesthetized with pentobarbital sodium, 30 mg/kg iv. A cuffed endotracheal tube was inserted, and respiration was controlled by positive-pressure ventilation. The carotid arterial pressure and lead II of the ECG were recorded. A midline laparotomy was performed, and a double-lumen catheter was introduced through a splenic vein. The end of the catheter was advanced into the portal vein to a point just proximal to the liver; its position was confirmed by digital palpation. Isoproterenol was infused through the distal lumen of the catheter, and portal venous blood was collected through the proximal lumen. Another catheter was introduced through the right external jugular vein and guided by palpation to lodge in a hepatic vein. Then the laparotomy incision was closed. A short, nonoccluding catheter was placed in the left femoral artery to sample arterial blood.

Following surgery, a 30-min period was allowed for stabilization. After a subsequent 30-min control period, *dl*-isoproterenol hydrochloride was infused at a rate of 0.4 μg/kg body wt/min, calculated as the base, into the portal vein for 30 min, using a syringe-driver pump. Samples of blood were collected simultaneously from the femoral artery, the portal vein, and the hepatic vein, after specified intervals during the control period, the infusion period, and for 30 min after the infusion was finished. The samples were placed in heparinized tubes and centrifuged immediately. Plasma [K] was measured using an internal-standard flame photometer. Based on the assumption of a blood volume equal to 7.9% of body weight (18), no more than 6.6% of the blood of any animal was

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rican Societies for Experimental Biology, Atlantic
.J., April 1978.

ısent address: Dalton Research Center, University
ouri, Columbia, Mo. 65211.
whom reprint requests should be addressed.

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removed during any experiment. The total volume of solution administered to each animal was 6.9 ml.

To assess the significance of change of plasma [K] in a given vascular bed during and after the infusion of isoproterenol, the differences between each experimental sample and the sample at min 0 were taken, and Student's *t* test for paired data was applied. To assess the significance of the difference of plasma [K] between two vascular beds at a given time during or after the infusion of isoproterenol, analysis of variance was used to determine if differences were present among the group means. Duncan's new multiple range test was used to determine which group means were significantly different.

Results. In six dogs that were infused intraportally with isoproterenol, the mean arterial blood pressure did not change significantly. The heart rate was significantly greater throughout the period of infusion ($P < 0.01$).

The temporal courses of the portal venous, arterial, and hepatic venous plasma [K] are shown in Fig. 1. The patterns of the three are qualitatively similar; in each vessel, the plasma [K] increased slightly, but not significantly, at min 2. The arterial plasma [K] decreased significantly at mins 10 and 50 ($P < 0.02$) and from mins 15 to 40 ($P < 0.01$). Although the hepatic venous plasma [K] decreased from mins 10 to 40 ($P < 0.01$), and it was slightly lower than the arterial plasma [K] during the infusion, the differences between the two were not significant ($P > 0.2$).

The portal venous plasma [K] decreased significantly at mins 15 and 20 ($P < 0.05$), and mins 25 and 30 ($P < 0.02$). Although the temporal courses of the changes in plasma [K] in the two vessels were similar, the plasma [K] did not decrease as much in the portal vein as in the hepatic vein. The hepatic venous plasma [K] was significantly lower than the portal venous plasma [K] at mins 4 ($P < 0.05$), 10 ($P < 0.01$), 15 ($P < 0.02$), 25 ($P < 0.02$), and 30 ($P < 0.05$).

During the infusion of isoproterenol, the portal venous plasma [K] slightly exceeded the arterial plasma [K], yet the differences were not significant.

Discussion. The plasma [K] during the control period in these experiments was lower than normal, perhaps due to the background

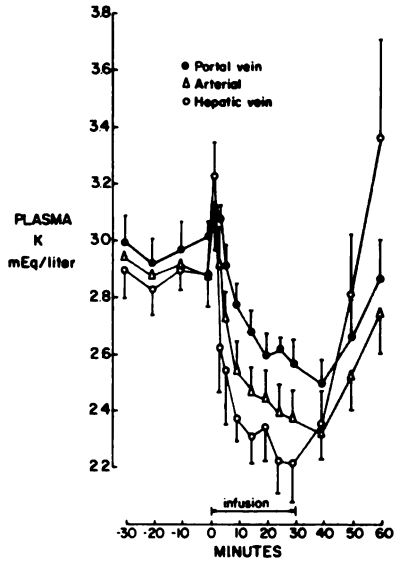

FIG. 1. Effects of constant infusion of isoproterenol into the portal vein on portal venous, arterial, and hepatic venous plasma [K]. Infusion at a rate of 0.4 μg/kg/min began at min 0 and ended at min 30. Mean ± SE of six experiments.

sympathetic activity, which was made greater by the surgery (19). Vick and Todd have suggested that normal sympathetic activity may exert an influence on the arterial plasma [K] (20). Since the initial increase in arterial plasma [K] caused by epinephrine is short lasting, sustained sympathetic function during stress is expected to act to reduce plasma [K]. Moreover, the use of pentobarbital as an anesthetic may have caused a decrease of the plasma [K] during the control period in the present experiments. Other investigators have observed a reduction in the plasma [K] during the use of a variety of anesthetics (21, 22), pentobarbital included (23).

It is assumed that the general response seen in the present experiments, which utilized *dl*-isoproterenol, would have been similar if *l*-isoproterenol had been used, although the magnitude of the response resulting from an equivalent dosage may have been different. During the period of hypokalemia caused by the infusion of isoproterenol, net movement of K from the plasma to the liver is indicated, as plasma [K] in the portal vein was significantly greater than that in the hepatic vein during most of the period of infusion. Although the differences between the arterial

ı [K] and the hepatic venous plasma re not statistically significant, there was ł toward a greater arterial concentra- ıssuming that plasma [K] in both the vein and the hepatic artery exceeded . the hepatic vein, these data establish e liver takes up K under the influence :nergic β-receptor stimulation, and that ɛr need not lose K immediately before- as occurs during the infusion of epi- ne, for the uptake to occur. However, ɔnceivable that increased sympathetic rge during surgery, as mentioned ear- ight have been associated with an ini- ease of K before the experimental pe- ɛgan.

ıough the differences between the ar- plasma [K] and the portal venous ı [K] during the infusion of isoproter- re not significant, the data indicate a release of K into the plasma in the teric bed at the same time that the liver up K. These opposing actions of the nd of the gut on the plasma [K] also ɐen observed during the period of hy- ːmia caused by systemic infusion of ɔhrine (13).

decrease of arterial plasma [K] during fusion of isoproterenol reflects the cu- ve uptake of K not only by the liver, ɔo by the skeletal muscle, if the isopro- l recirculates sufficiently. The concen- ı of the drug which reached skeletal ː beds is not known. Apparently, not h of the isoproterenol escaped inacti- by the liver to decrease mean arterial re, although enough of the drug d the heart to increase the heart rate ; the period of infusion.

ːr the infusion of isoproterenol was ɛted, the hepatic venous plasma [K] led the plasma [K] in the portal vein the hepatic artery, which suggests that, the influence of isoproterenol, the liver ıp more K than it could retain. This w of K from the liver would act to ː arterial plasma [K] to a normal level. ɔther investigations, isoproterenol has shown to cause an early increase in l plasma [K] (14, 24). The hyperka- ɛffect is not constant, and the response igible in some animals, even with high Todd and Vick (15) reported no significant increase of mean arterial plasma [K] in dogs infused with isoproterenol at a rate of 2 μg/kg/min in the femoral vein. In the present experiments, although a slight increase of plasma [K] occurred at min 2, it was not significantly different from the control value.

Intraportal infusion of isoproterenol at the rates of 0.1 to 0.4 μg/kg/min does not increase the portal venous blood flow significantly (25) (unpublished observations), although other investigators (26–28) have found that isoproterenol dilates the hepatic arterial vessels. Effects on the intrahepatic portal venous bed are unclear. Hanson (26) found an inconsistent response of the portal vessels to the isoproterenol which was infused through the hepatic artery, and, portal vessels dilated in some perfused dog liver preparations *in situ*, but in some others, they did not. Green (29) suggested that pentobarbital sodium, used for anesthesia, may have diminished the resting tone of the intrahepatic portal vessels, and, thus, prevented further dilation caused by the isoproterenol.

The mechanism by which β-receptor agonism causes the uptake of K by the liver is not known. Since the liver need not lose K beforehand, which might establish a passive gradient for the uptake to occur, we need to postulate some type of active transport mechanism that is stimulated by isoproterenol. Dockry *et al.* (30) have suggested that isoproterenol causes muscle to take up K by stimulating an electrogenic Na "pump." Porte (31) demonstrated that isoproterenol can stimulate the pancreas to secrete insulin. Although insulin can lower plasma [K] (32), Pettit and Vick (33) found that the actions of the catecholamines on the plasma [K] do not depend on the release of pancreatic insulin. These authors postulated that the β-receptor activity might increase the amount of insulin the pancreas secretes, while simultaneously counteracting the peripheral action of insulin on tissue uptake of K.

Summary. Constant infusion of isoproterenol into the portal vein of the dog produces a sustained decrease of the plasma [K] in the femoral artery, the hepatic vein, and the portal vein. During the hypokalemia, the hepatic venous plasma [K] falls significantly below the portal venous plasma [K], which indicates a net uptake of K by the liver; at the same

time that the liver takes up K, the gut releases a slight amount of K. A preceding loss of K by the liver is not essential to the occurrence of the uptake; therefore, the data suggest that isoproterenol acts directly to increase the uptake of K by the liver.

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Pineal Melatonin Content throughout the Hamster Estrous Cycle (40649)[1]

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reproductive system of adult Syrian ers is extremely sensitive to photope- effects (1). Upon exposure to short eriods female hamsters experience a tic decrease in uterine weight (2) and to exhibit vaginal changes associated strous cyclicity (3). Twenty to twenty- eeks after initiation of short photope- conditions gonadal function returns, f there has been no change in photo- ic cues (2). At this time hamsters are ory toward short photoperiods and re- refractory until an intervening long eriodic exposure of at least 10 weeks curred (4, 5). The response of hamsters rt photoperiods is dependent upon an pineal gland (2). If the pineal gland is ed prior to exposure of hamsters to hotoperiods the gonads do not regress, as, if gonadal regression has already ed, pinealectomy results in a rapid re- n reproductive function. Daily injec- of melatonin, a putative pineal hor- can induce gonadal regression in pin- mized hamsters (6, 7). Consequently, nin is considered by many investiga- be the pineal factor which mediates eriodic effects upon the reproductive of this species. Recently, it has been ed that melatonin injections induce l acyclicity in hamsters when admin- on the evening of proestrus of each for five cycles but not when adminis- on the evening of diestrus (8). Since btained from rats indicate that less nin is found in urine collected on Day estrus) of the estrous cycle (9), it could pothesized that in long photoperiodic ions the hamster pineal gland is in- l by the preovulatory surge in estrogen on and that in short photoperiodic conditions the pineal gland becomes insensitive to estrogen feedback, an escape which results in gonadal regression. To test the validity of this hypothesis we have quantified pineal melatonin content throughout the estrous cycle of female hamsters maintained in a 14-hr-light:10-hr-dark photoperiod. If the hypothesis is correct, we would expect to observe a decrease in pineal melatonin content on the day preceding ovulation when estrogen concentrations are at a maximum.

Materials and methods. Adult female hamsters, 100–120 g, obtained from Lakeview Hamster Colony (Newfield, N.J.) were housed in a room with overhead fluorescent lighting (Sylvania Super Saver 35, F40W/RS/SS). Lights were turned on automatically at 0600 hr and off at 2000 hr (14-hr-light:10-hr-dark). Food and water were supplied *ad libitum*. Progression through 4-day estrous cycles was monitored for each hamster for a period of 1 month (10). At the end of this interval, 32 groups of seven to eight hamsters each were decapitated sequentially at 1000, 1400, 1800, 2200, 2400, 0200, 0400, and 0600 hr for 4 consecutive days. Hamsters were chosen so that representatives from each day of the estrous cycle were present at each sacrifice time. Those decapitations occurring at night were performed with the aid of a dim red light (25-W tungsten bulb behind a No. 1A Safelight filter, Kodak). Following decapitation, pineal glands were collected and placed into individual 1-ml aliquots of chilled 0.1 *M* sodium phosphate buffer, pH 7.0, containing 0.9% sodium chloride. The pineal glands were sonicated and stored as homogenates at −20°. Three weeks later, melatonin concentrations were quantified by radioimmunoassay of duplicate 200-μl aliquots from each 1-ml homogenate (11). Pineal melatonin concentrations were compared by analysis of variance.

Results. The quantities of melatonin in female hamster pineal glands throughout the

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estrous cycle are presented in Fig. 1. Values represent the mean ± SEM for eight hamsters during times of darkness and for seven hamsters during times of light. In the four radioimmunoassays utilized to obtain these values, control samples estimated to contain 171, 334, and 1006 pg/ml had respective intraassay coefficients of variation averaging 12.7, 6.8, and 9.6%. The respective interassay coefficients of variation were 14.9, 9.3, and 12.3%. The mean quantity of immunoreactivity in a 1 ng/ml solution of melatonin in assay buffer ranged between 951 and 1142 pg/ml. The least detectable concentration of melatonin ranged between 3 and 34 pg/ml.

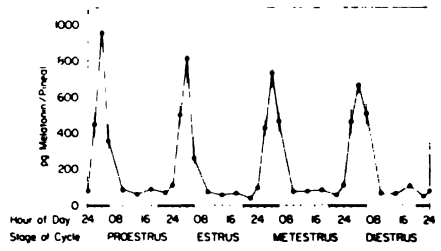

FIG. 1. Pineal melatonin content throughout the hamster estrous cycle. Values represent the mean ± SEM for eight hamsters during times of darkness and for seven hamsters during times of light. The solid bars on the time axis represent darkness in successive 14-hr-light:10-hr-dark photoperiods.

On each day of the estrous cycle a distinct rhythm in pineal melatonin content was observed. Mean melatonin concentrations ranged between 37 and 112 pg/gland throughout the day and during the first 4 hr of darkness. The highest mean melatonin concentrations, which ranged between 669 and 954 pg/gland, occurred at 0400 hr, 2 hr prior to the onset of light. Comparison of melatonin concentrations in pineal glands obtained at 0600 hr, just prior to light on, with those in pineal glands obtained at 0400 hr yielded a significant difference ($P < 0.001$). No significant difference could be found between values obtained at a particular time of day as a function of day of the estrous cycle. In particular, the F value obtained from analysis of samples collected at 0400 hr was 1.09 ($P > 0.2$).

Discussion. The results presented in this communication indicate that pineal melatonin production in hamsters is not inhibited by estrogen feedback in long photoperiodic conditions. Consequently, darkness-induced gonadal regression in hamsters cannot be explained by hypothesizing that short photoperiodic conditions result in an escape of the pineal gland from estrogen inhibition of melatonin synthesis. The data do not exclude hypotheses which depend upon 4-day cyclic changes in pineal melatonin production in hamsters maintained in short photoperiods. The lack of a difference in pineal melatonin production on different days of the hamster estrous cycle is similar to the results reported for sheep (12), but opposite to those reported for rats (9, 13) and humans (14). The reason for this difference is unknown. It is possible that pineal melatonin content, as reported here, does not accurately reflect secretion. This possibility seems unlikely since no data are available which suggest that melatonin is either stored or degraded within the pineal gland. Until such storage or degradation is demonstrated it can be assumed that all melatonin produced within the pineal gland is secreted and that pineal melatonin content accurately reflects secretory rates. This assumption is supported by data obtained from rats showing that pineal and serum melatonin concentrations have parallel peaks and troughs (15), and that photo-induced reduction in pineal melatonin content is closely paralleled by reduction in serum melatonin concentrations (16).

The circadian rhythm in pineal melatonin content of female hamsters reported in this study is of similar magnitude and duration as that reported for male hamsters maintained in the same photoperiodic conditions (17) and for female hamsters maintained in an 8-hr-light:16-hr-dark photoperiod (unpublished observations). The decrease in pineal melatonin content prior to the dark-to-light transition was found in male hamsters (17) and has been reported for rats (15). This decrease in pineal melatonin concentrations prior to exposure to light supports the contention that melatonin production is a consequence of an endogenous circadian rhythm.

Summary. A circadian rhythm in pineal melatonin content persisted throughout the estrous cycle of female hamsters maintained in a 14-hr-light:10-hr-dark photoperiod. Peak melatonin concentrations occurred 8 hr after the onset of darkness and were of similar

duration and magnitude on each day of the reproductive cycle. These results do not support the hypothesis that pineal melatonin production in hamsters is inhibited by estrogen feedback.

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In vitro Properties of FBR Murine Osteosarcoma Virus (40650)

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We have previously reported the isolation of two murine osteosarcoma viruses, both having Type C morphology. FBJ virus was derived from a spontaneous osteosarcoma occurring in a CF No. 1 mouse (1) and FBR virus from a ^{90}Sr-induced osteosarcoma in an X/Gf mouse (2, 3). Since their isolation, FBJ-MuSV[2] and FBR-MuSV have been maintained by continuous cell-free passage in their respective host strains. Currently at their 102nd and 67th passages, respectively, both viruses are potent inducers of parosteal osteosarcomas. Intraperitoneal injection of either virus into newborns of its respective host strain results in tumors in as early as 3 weeks with a final incidence of 100%. These two viruses are biologically distinct: Whereas each is highly tumorigenic in its own strain, FBJ-MuSV is poorly tumorigenic in X/Gf mice and FBR-MuSV is poorly tumorigenic in CF No. 1 mice (2, 3).

FBJ and FBR, together with the Gazdar (4) and BALB-MuSV (5) comprise the few natural MuSV isolates. Other MuSVs were recovered from sarcomas resulting from the injection of leukemia virus into mice (6, 7) and rats (8, 9).

The specific osteosarcomagenic property of FBJ-MuSV and FBR-MuSV is unique among murine sarcoma viruses as none of the other isolates induces bone tumors in mice (10). However, the Moloney isolate can induce osteosarcomas if injected directly into the bone marrow of rats (11–13).

In this report we describe the properties and propagation of the FBR isolate in tissue culture. Some of the biological properties of the FBJ isolate in tissue culture have been reported earlier (14–16).

Materials and methods. Viruses. A pool of FBR virus (Pool No. 77-1) was prepared from 10 g of osteosarcomas that had been induced by tumor-derived FBR virus injected into newborn X/Gf mice (Animal Passage No. 54). The tumors were homogenized (MicroMill, Chemical Rubber Company, Cleveland, Ohio) and extracted with 100 ml of Hank's balanced salt solution (GIBCO). The extract was clarified (International High-Speed refrigerated centrifuge, rotor No. 856, 9000 rpm, 10 min), filtered (Millipore, Millex 0.45 μm), and stored in liquid nitrogen in 1-ml aliquots in sealed glass ampules. The tumorigenicity of Virus Pool No. 77-1 was tested by injecting 0.1 ml of the preparation into the abdominal cavity of X/Gf newborns. The undiluted pool produced multiple osteosarcomas in all 12 recipients in 25–28 days. A 1:5 dilution produced multiple osteosarcomas in all 14 animals in 32–57 days.

Virus from tissue culture sources was obtained from 1-day-old spent media collected from confluent FBR-MuSV(MuAV)-infected cultures. The media were clarified (clinical table centrifuge) and filtered (Millipore, Millex, 0.45 μm).

Cells. Established cell lines were obtained from the following sources: Dr. R. H. Bassin (National Cancer Institute), D152, D55, Sc-1, NIH/3T3; Dr. P. Fischinger (National Cancer Institute), XC, BALB/c-3T3; Dr. G. Todaro (National Cancer Institute), E36, 64, 8155, 88; Dr. J. Stephenson (National Cancer Institute), NRK, sheep (SL). Cell lines BC352, A5700, BE849, AP737, AM237, and A0134 were supplied by the Naval Biomedical Research Laboratory, Oakland, California, under a contract from the Division of Cancer Cause and Prevention, National Cancer Institute.

Secondary whole mouse embryo cultures were derived from mouse embryo primary

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[2] Abbreviations used: MuSV, murine sarcoma virus; MuAV, murine-associated virus; ffu, focus-forming unit; pfu, plaque-forming unit.

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of NIH Swiss, C57BL/6, and BALB/
rom the colonies maintained at Ar-
ational Laboratory.
ltures were grown in plastic dishes or
'alcon Plastics) and kept in 37° hu-
CO_2 incubators in McCoy's 5A me-
IBCO) supplemented with 100 μg/
tomycin and 100 units/ml penicillin,
mine, and 10% heat-inactivated fetal
um (56°, 30 min). Media were
every 2–3 days and cultures were
red with trypsin every 5–7 days.
pplements used in some experiments
ptose phosphate broth (GIBCO), po-
(Aldrich), and dexamethasone

assay. Transformation assays for sar-
uses (17) were routinely carried out
indicator cells; exceptions in some
nts are specified. Falcon plastic grid-
i dishes (60 mm) were seeded with
per dish. Twenty to twenty-four
ter, the cultures were treated with 2
edium containing 25 μg/ml DEAE-
(Pharmacia) without serum. After a
ncubation, the dishes were drained
im and the cells were washed with
containing no serum. Duplicate
ere then infected with 0.2 ml serial
lilutions of virus inocula (10^{-1}, 10^{-2},
$^{-4}$). After 1 hr incubation, 4 ml of
medium was added. All cultures
fresh media every 2–3 days. Foci of
ned cells were counted under an in-
ght microscope on Days 10–14 post-
. Titers were given in focus-forming
(ffu/ml) of undiluted virus inocu-

assay. Standard XC plaque assays
associated nontransforming viruses
tinely carried out on duplicate D55
which were infected as described
cept with a wider range of virus
. On Day 6 postinfection, all cultures
diated with ultraviolet light (G. E.
lal Lamp, 1500 ergs/mm^2) and
with 10^6 XC cells per dish. When the
reached confluency in 3–4 days, the
ltures were fixed and stained with a
of 1% methylene blue in methanol,
thanol, and 1% carbol fuchsin in
l in a ratio of 3:2:1, respectively.
s centers were counted under a di-
secting microscope and expressed as pfu/ml of undiluted virus inoculum.

Polymerase assay. Various cell cultures were tested for virus by carrying out reverse transcriptase assays on high-speed pellets (1 hr at 100,000 *g*) obtained from preclarified media (10 min at 10,000 *g*). After draining, each pellet was resuspended in 120 μl of preincubation buffer (0.01 *M* Tris, pH 8.2, 0.01 m*M* dithiothreitol, and 0.33% nonionic detergent NP40), and incubated for 10 min on ice. A 50-μl aliquot of the suspension was assayed with 50 μl of reaction mixture containing a final 100-μl reaction volume: 0.05 *M* Tris, pH 8.2, 0.01 *M* NaCl, 2 m*M* dithiothreitol, 50 μ*M* [^{3}H]dGTP (ICN, diluted to 4 Ci/mmole), 25 μg/ml oligo(dG)$_{8-12}$:poly(rC) (P. L. Biochemicals). For control, a matched 50-μl aliquot of the resuspended pellet was incubated with an equal volume of the same reaction mixture but without oligo(dG)$_{8-12}$: poly(rC). After incubation at 37° for 1 hr, both test and control reactions were terminated with 20% cold trichloroacetic acid. Acid-precipitable products were collected on glass fiber circles (Whatman GFC), dried under an infrared lamp, and assayed in a liquid scintillation counter. A sample was considered positive when the tritium incorporation of the test assay was more than twice that of the control assay.

Cell cloning. Sc-1 cells, chronically infected with FBR virus from Pool No. 77-1, were cloned by an endpoint dilution technique in microwell plates (Tissue Culture Cluster 96, Costar). Of 30 wells that were seeded, 11 had no cells, 14 had single cells, and 5 had more than one cell. Clones 1–5 were derived from the single-cell wells.

Results. Host range studies. Sixteen normal cell lines derived from a wide range of animal species were inoculated with serial 10-fold dilutions of FBR virus, Pool No. 77-1. Infectivity was monitored by examination for morphologic transformation of the treated cultures and by focus, plaque, and reverse transcriptase assays on culture media. FBR virus proved to be infectious for all murine cells including those of rat (Table I). However, cells from 10 different nonmurine species, including human, were totally resistant to infection by this virus. After two passages 4–5 weeks postinfection, all infected cultures

TABLE I. HOST RANGE OF FBR-MuSV(MuAV)

Species (cell line)	Initial infected cultures: Focus assay[a] (ffu/ml)	Initial infected cultures: Virus detection[b] Focus assay (ffu/ml)	Plaque assay (pfu/ml)	Polymerase assay[c]	Infected cultures at passage two: Virus detection[d] Focus assay (ffu/ml)	Plaque assay (pfu/ml)	Polymerase assay
Mouse (NIH 3T3)	5.0×10^2	1.0×10^2	2.5×10^2	+	5.5×10^2	1.5×10^4	+
Mouse (D152)	6.4×10^5	1.0×10^5	2.1×10^7	+	4.0×10^5	4.7×10^7	+
Mouse (Swiss D55)	1.2×10^5	1.5×10^4	3.6×10^7	+	1.5×10^4	1.2×10^6	+
Mouse (Feral Sc-1)	***	2.0×10^4	1.5×10^6	+	1.0×10^4	2.9×10^7	+
Rat (OM—NRK)	1.5×10^2	0	1.5×10^5	+	0	2.0×10^5	+
Chinese hamster (E36)	0	0	0	−	0	0	−
Mink (64)	0	0	0	−	0	0	−
Sheep (SL)	***	0	0	−	0	0	−
Rabbit (BC352)	0	0	0	−	0	0	−
Rabbit (A5700)	0	0	0	−	0	0	−
Cat (BE849)	0	0	0	−	0	0	−
Dog (8155)	***	0	0	−	0	0	−
Bat (88)	0	0	0	−	0	0	−
Cow (AP737)	0	0	0	−	0	0	−
African green monkey (AM237)	0	0	0	−	0	0	−
Human (A0134)	0	0	0	−	0	0	−

[a] In the cases indicated by *** transformation could not be assessed because of the detachment of the cells, whether infected or uninfected, prior to the time required for the development of visible foci in permissive host cells.
[b] Culture media were tested for presence of virus by the indicated assays 8–12 days postinfection.
[c] For the polymerase assay (+) = a positive reaction (−) = a negative reaction.
[d] Media were tested 3–6 days after second passages.

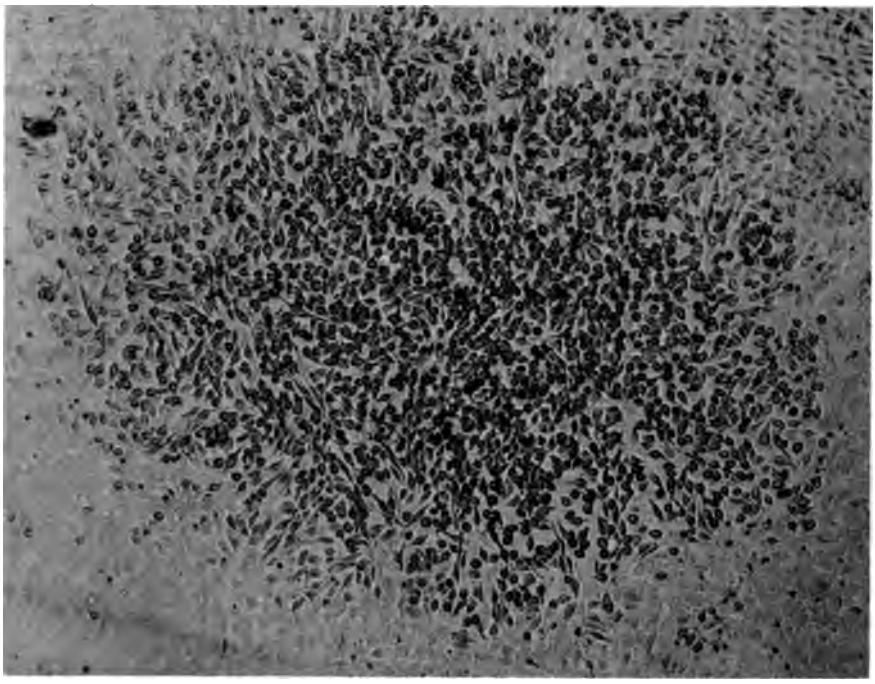
were retested. Results comparable to the first test were obtained.

Transforming and nontransforming activities. In appropriate murine cells, such as BALB/c (A31, D152) and Swiss (D55), FBR virus readily induce morphologic transformation. With diluted virus inoculum, discrete foci of disoriented, refractile, rounded, and spindle-shaped cells were visible (Fig. 1). Whereas similar foci induced by other MuSVs usually appeared on Days 5–6 (8), those induced by FBR virus appeared later. In infected BALB/c monolayer cultures, recognizable FBR foci first appeared on Days 7–8 postinfection and were best quantitated on Days 10–14. On standing, these foci continued to enlarge and overlap one another. Totally transformed cultures often resulted. Attempts to speed up focus development with a polybrene medium supplement and high serum concentrations were unsuccessful.

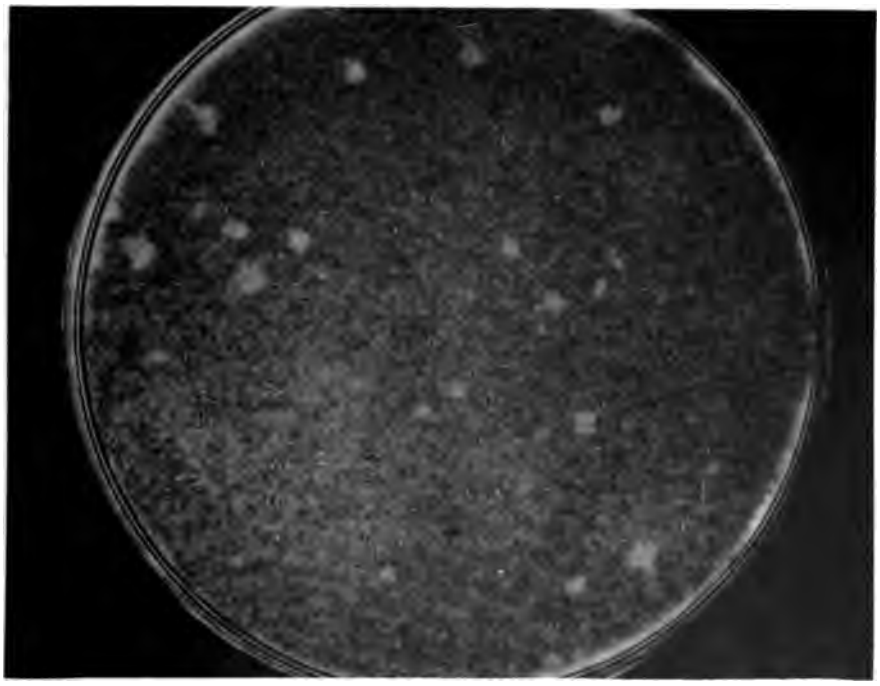
For focus assay, D55 and D152 are the best indicator cells. They yield the highest transforming titers (Table I) and the most stable foci, and their monolayers remain smooth and attached for more than the required 2-week period. Focus induction assay using NIH/3T3 cells is not satisfactory as these monolayer cultures tend to lift early. Even with low initial cell densities and low serum concentrations, NIH/3T3 cells reach confluency rapidly and usually detach before Day 7 and prior to the usual time required for the development of visible foci in susceptible indicator cells. FBR virus induces foci in rat NRK cells that are morphologically different from those induced in mouse cells. Rat foci appear on Days 6–7 postinfection and are small and transient, consisting of clusters of small, rounded-up cells that detach easily.

As with all other mammalian sarcoma virus isolates, FBR-MuSV has an associated nontransforming virus detectable by a standard infectious center plaque assay with XC cells (Fig. 2). This associated virus, FBR-MuAV, is present in two to three logs higher concentration than the transforming component (Table I).

Focus titration assays of FBR-MuSV-(MuAV) consistently yielded single-hit patterns whether foci were counted early (Day 9) or late (Day 15). Single-hit kinetics was also observed whether the infecting virus



. 1. Focus of transformed cells induced by FBR-MuSV(MuAV) on D55 cells. Photograph shows a single entative focus of morphologically altered cells 14 days postinfected in a plate infected with virus inoculum at) dilution, × 170.24.



. 2. Syncytial plaques formed between XC cells and irradiated D55 cells infected with FBR-MuSV(MuAV).)55 cells were infected with a 10^{-4} dilution of virus inoculum (Pool No. 77-1), × 2.66.

stock had a one, two, or three log excess of the nontransforming component (Fig. 3).

Tropism of FBR-MuSV(MuAV) was determined by comparing the susceptibilities of embryonic cells from mice having different alleles in the Fv-1 gene locus (19–21). FBR-MuSV(MuAV) proved to be b-tropic, showing higher infectivity for b than n cells (Table II). The differences in titers are of the order of two logs for the transforming virus and four logs for the nontransforming virus.

Virus propagation. To determine the best virus–cell system for the large-scale production of FBR-MuSV(MuAV), the transforming and nontransforming titers of viruses produced by various infected murine cell lines were compared. Results of representative experiments are shown in Table III. In three separate trials, infected NIH/3T3 cells yielded low titers of transforming virus, and the transforming titer of chronically infected cells tended to decrease with passage. On the other hand, the nontransforming titer produced by NIH/3T3 cells was fairly high and increased with cell passage to a stable high titer of 10^6–10^7 pfu/ml. Thus, NIH/3T3 cells preferentially replicate the nontransforming component and are suitable for the propagation of FBR-MuAV. Chronically infected BALB/c and Sc-1 cells yielded much higher titers of the transforming component. However, each also produced a two log excess of the nontransforming virus. Attempts to increase virus titers of infected Sc-1 cells by supplementing the media with tryptose phosphate broth, DEAE-dextran, polybrene, or high serum resulted in only minimal improvement. Dexamethasone and 10% conditioned medium did affect a significant but small enhancement (approximately one log). The high virus-producer, FBR-infected Sc-1 cells, were cloned by endpoint dilution in microplates, and the virus titers produced by the single-cell derivatives were compared (Table IV). Of the five clones tested, clone 1 yielded the best ratio of transforming to nontransforming virus. This clone was selected for large scale virus production in roller bottles. Periodic testing showed that virus titers of the clone 1 roller cultures remained essentially unchanged for more than 10 months. In soft agar, these cells have a cloning efficiency of almost 100%.

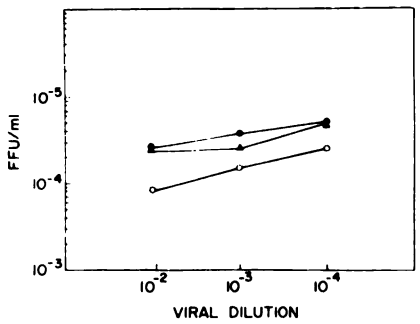


FIG. 3. Focus titrations of FBR-MuSV(MuAV) stocks having varied ratios of transforming to nontransforming viruses. Standard assays were carried out on virus preparations derived from different clones of chronically infected Sc-1 cells. The corrected titers in ffu/ml are plotted against virus dilutions. (○) clone 1, ffu:pfu—1:10; (▲) uncloned, 1:200; (●) clone 5, 1:7,500.

TABLE II. TROPISM OF FBR-MuSV(MuAV)[a]

Strain	Fv-1 genotype	Focus titer (ffu/ml)	Plaque titer (pfu/ml)
NIH Swiss	n	9.2×10^1	5.0×10^2
C57BL/6	b	1.6×10^4	4.2×10^6
BALB/c	b	1.5×10^4	2.8×10^6

[a] FBR virus Pool No. 77-1 was titrated on various secondary embryo cultures derived from mice having different Fv-1 genotypes. Culture dishes were seeded with 3×10^5 cells and inoculated in quadruplicate with serial 10-fold dilutions of the virus. On Day 7, two plates of each dilution were irradiated for XC plaque assay. The matched plates were held for focus counting on Days 10–14.

TABLE III. TRANSFORMING AND NONTRANSFORMING VIRUS TITERS OF CHRONICALLY INFECTED MURINE CELL LINES

Cell line	Focus titer (ffu/ml)	Plaque titer (pfu/ml)
NIH Swiss—3T3	5.0×10^1	2.0×10^5
BALB/c—D152	1.0×10^5	2.0×10^7
Swiss—D55	1.4×10^4	3.0×10^7
Feral—Sc-1	2.5×10^4	1.5×10^6

Discussion. This report describes the properties and propagation of FBR-MuSV-(MuAV) in tissue culture and contains basic information useful for further characterization of this interesting virus. FBR-MuSV(MuAV) is apparently one of the few available authentic murine endogenous sarcoma viruses. Its origin and unique osteosarcomagenic property may provide a model for the study of the role of this virus in ^{90}Sr-

d osteosarcomas.
t-MuSV(MuAV) derived from virus-
d bone tumors in X/Gf mice appears
trictly ecotropic. Although only a lim-
ımber of tissue cells from a variety of
ıalian species were tested, the data
showed that the virus is infectious for
: cells only. All infected cultures were
ɔred for morphologic transformation
ıeir spent media tested for virus by
: transcriptase, focus induction, and
aque assays. There was no evidence of
ɔn of the nonmurine cells even on re-
after two cell passages 4–5 weeks post-
ɔn. These negative data for nonmurine
lso indicate the absence of readily de-
e xenotropic (22) or polytropic viruses

ıor-derived virus preparations consist
ınsforming MuSV and a nontransform-
uAV. When tested in various mouse
o secondary cultures, both the trans-
ıg and nontransforming activities
d distinct b-tropism. This fact is con-
with the finding that the X/Gf mouse
v-1^b genotype (J. W. Hartley, personal
ınication), which is restrictive for n-
viruses.

focus-inducing behavior of FBR-
(MuAV) is consistently single-hit. This
hit pattern may be due to: (i) the pres-
f excess helper virus, (ii) competence of
coma virus, or (iii) late quantitation of
he first explanation is unlikely since
ne pattern was observed whether the
of MuAV over MuSV was one, two,
ee logs. The second would be most
ting but it has no precedent. The third
ely possibility. In contrast with other
of MuSV that induce foci within 5–6
ecognizable FBR-induced foci first ap-

IV. TRANSFORMING AND NONTRANSFORMING
S TITERS PRODUCED BY VARIOUS CLONES OF
FBR VIRUS-INFECTED Sc-1 CELLS

	Focus titer (ffu/ml)	Plaque titer (pfu/ml)	Ratio (ffu:pfu)
	2×10^4	2×10^5	1:10
	5×10^4	4×10^6	1:80
	2×10^3	6×10^5	1:300
	2×10^4	1×10^6	1:50
	8×10^4	6×10^8	1:7, 500
d	1×10^4	2×10^6	1:200

pear 7–8 days after infection and are not sufficiently well formed for quantitation until 10–14 days. Perhaps FBR foci develop primarily by cell division and not by cell recruitment involving infection of adjacent cells by progeny virus (10, 24–26). Consequently, in 2 weeks all transformed clones, whether originally infected with or without helper virus, will have grown to a sufficient size to be scored. These focus-inducing properties of FBR virus are similar to those of FBJ-MuSV(MuAV) in terms of single-hit kinetics and late appearance of foci (14).

Further immunologic and biochemical characterizations, especially of sarcoma-specific sequences, require large quantities of sarcoma-rich virus. For this purpose an appropriate virus–cell system was selected for mass culture. A clone of infected Sc-1 cells produces high titers of the transforming virus (10^5 ffu/ml) without a vast excess of the nontransforming component (10^6 pfu/ml). This clonal cell line grows well in roller bottles and remains a good producer of sarcoma-rich virus. These cells are themselves completely transformed as indicated by their high cloning efficiency in soft agar. The viruses they produce are highly transforming *in vitro* and are potent inducers of osteosarcomas *in vivo*. Thus, tissue-culture-derived FBR-MuSV(MuAV) has biological properties similar to its parent virus except for an enrichment in the transforming component.

Summary. This report describes the *in vitro* biological properties of an osteosarcoma virus, FBR, which was originally isolated from a ^{90}Sr-induced osteosarcoma in an X/Gf mouse. The virus complex consists of a transforming component, MuSV, and a nontransforming component, MuAV. Foci of cells transformed by FBR-MuSV(MuAV) are late appearing and consistently yield single-hit focus titration patterns. The isolate is infectious for only murine cells and shows distinct b-tropism. A clone of transformed cells yielding high-titered sarcoma-rich virus has been propagated for the large-scale production of this virus. Tissue-culture-derived FBR-MuSV(MuAV) possesses osteosarcomagenic properties comparable to those of the parent virus.

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and Pituitary Concentrations of Growth Hormone and Prolactin in Pygmy Mice[1] (40651)

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it pygmy (*pg/pg*) mice first described in 1950 (1) exhibit marked growth on, growing to only about half the ormal littermates at 6 weeks of age ough heterozygotes reproduce nor- he homozygotes of both sexes are ifertile or sterile.

mutation is not completely recessive terozygous *Pg/pg* mice tend to be it smaller than homozygous *Pg/Pg*), Wolff, unpublished data). Hetero- *g/pg* females also tend to have some- aller litters than *Pg/Pg* dams (Wolff, hed data).

ause of pygmy's growth retardation own. Based on pituitary transplan- periments, King (2) postulated that l size of the mouse was not due to ormone (GH) deficiency, but rather sponsiveness of the body tissue to noin and Richmond (3) reached the nclusion after unsuccessful attempts ating body growth of these mice by porcine GH, and suggested that the nouse could serve as a model of the African pygmy. GH levels in the se- pituitary gland of pygmy mice have measured.

study, we measured GH and pro- RL) in pygmy mice of both sexes. nother pituitary hormone known to body growth in dwarf mice (4). RL concentrations were determined sal conditions as well as after treat- pygmy mice with the PRL release perphenazine. In addition, we have ted the effects of PRL administra- ody weight gain of these mice.

ted by Grant CA-18664, awarded by the ancer Institute, DHEW. This is Publication n the Lutcher Brown Center for Diabetes and ogy.

Materials and methods. Pygmy (*pg/pg*) mice were produced by mating heterozygous VY/Wf-*Pg/pg* females with C3H/HeNIcrWf-*Pg/pg* males. Normal *Pg/Pg* (VY/Wf × C3H/HeNIcrWf) F-1 hybrids were used as controls. After weaning at 4 weeks of age, all mice were shipped by air from Jefferson to La Jolla. There they were kept in 36 × 30 × 18-cm plastic cages, 10–12 normal or 18–20 pygmy mice per cage, in a light (12-hr light:12-hr darkness)- and temperature (24 ± 1°)-controlled room. Wayne Lab Blox (6% fat) and tap water were available *ad libitum*. At approximately 90 days of age, control and pygmy mice of both sexes were decapitated within 30 sec after removal from the cage. Blood and pituitary glands were collected individually from groups of 17–19 animals. They were stored at 4° not longer than 2 hr before processing. Each serum was separated by centrifugation and each pituitary gland was weighed and extracted with 0.05 *M* Na_2CO_3–$NaHCO_3$ buffer, pH 10, as described earlier (5). GH and PRL were measured in duplicate samples by homologous radioimmunoassays specific for each hormone. The details of the assay procedure have been described previously (6, 7). The biological potency of the mouse prolactin standard used was 25 IU/mg and that of the mouse GH, 3.1 USP U/mg.

To determine their responses to stimulated release of PRL, pygmy and normal mice of both sexes were given single intraperitoneal injections of 1 μg perphenazine/g body wt. They were killed by decapitation at 0, ½, or 1 hr after treatment and serum prolactin concentrations were determined.

For growth stimulation study, a separate group of male and female pygmy mice were used. Beginning at 40 days of age and continuing for 8 weeks, half of the mice of each sex were injected subcutaneously twice daily (at

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8:30 AM and 4:30 PM) with 50 μg of ovine PRL (NIH-P-S_{12} Ovine; potency 35 IU/mg). The remaining mice were injected with equal volumes of saline solution (0.85% NaCl). The hormone was dissolved in saline to a concentration of 1 mg/ml. The addition of about 50 μl 1 *N* NaOH per 20 ml of PRL solution helped to dissolve the PRL. Body weights of the mice were recorded twice a week.

The results were analyzed statistically with a two-way analysis of variance.

Results. Pygmy mice weighed about 60% less than normal controls at 3 months of age (Table I). Pituitary glands from pygmy mice were significantly lighter than those from comparable normal controls ($P < 0.01$). The pituitary glands of male pygmies averaged only 29% as heavy as those of the normal males, while those of female pygmies were only about 21% as heavy as glands from normal females. The serum concentrations of GH and PRL in pygmy mice of both sexes were similar to those in controls ($P > 0.05$). However, GH and PRL concentrations in the pituitaries of pygmies were much lower than in the pituitaries of normal mice ($P < 0.01$). Among the males, pituitary GH concentration of the pygmy mice was only about 41% as great as that of the normal mice and PRL content was only 18% as high. Among pygmy females, the pituitary GH concentration averaged 49% and the pituitary PRL concentration was about 15% of the comparable values in normal female mice.

As expected, a single injection of perphenazine induced the release of large amounts of PRL by the pituitary glands of normal mice ($P < 0.01$) (Table II). In contrast, the pituitary glands of the pygmy mice did not respond to treatment with perphenazine ($P > 0.05$).

Daily administration of 100 μg of ovine PRL for 8 weeks did not enhance body weight gain of male pygmy mice appreciably ($P > 0.05$) (Fig. 1). However, in females, a slight increase was observed. The net gain in body weight of PRL-treated females was 20% higher than that of saline-treated controls ($P < 0.01$).

Discussion. While basal serum levels of GH and PRL in dwarf (*dw/dw*) mice have been reported to be clearly subnormal (8), the results of the present study demonstrate that, in the pygmy mouse, the basal serum concentrations of immunoreactive GH and PRL are in the normal range. In contrast, GH and PRL levels in the pituitary gland of pygmy mice and serum PRL levels after a provocative challenge were markedly subnormal. The lack of pharmacologic means of inducing GH release in the mouse has prevented us from performing a stimulatory test for GH release. However, since the pituitary contents of both PRL and GH were quite low, the release of GH may also be expected to be deficient. It is thus possible that the pulsatile secretion of GH and PRL (9), which is the normal mode of secretion for many pituitary hormones, may be lacking or deficient in the pygmy mouse. It should be noted that in the human African pygmy, GH levels were found to be normal not only under basal but also under stimulated conditions of release (10, 11).

The disproportionately undersized pituitary, the marked deficiency of the glandular GH and PRL contents, and the lack of PRL release in response to perphenazine all point to a defect of the pituitary–hypothalamic system in the pygmy mouse. Whether the growth retardation of the pygmy is due to a subresponsiveness of the body tissue to GH, as

TABLE I. PITUITARY AND SERUM CONCENTRATIONS (MEAN ± SEM) OF PROLACTIN AND GROWTH HORMONE IN 3-MONTH-OLD PYGMY (*pg/pg*) AND NORMAL (*Pg/Pg*) (VY/Wf × C3H/Wf) F-1 HYBRID MICE

Sex and phenotype	No. of mice[a]	Body wt (g)	Pituitary gland wt (mg)	Prolactin		Growth hormone	
				Serum (ng/ml)	Pituitary (μg/mg)	Serum (ng/ml)	Pituitary (μg/mg)
Male							
Normal	19	32.5 ± 0.6	2.01 ± 0.04	10.5 ± 0.7	1.68 ± 0.04	62.9 ± 11.9	121.7 ± 3.8
Pygmy	19	13.2 ± 0.2*	0.58 ± 0.01*	13.2 ± 1.2	0.30 ± 0.02*	45.6 ± 10.1	49.7 ± 1.6*
Female							
Normal	19	24.3 ± 0.2	2.17 ± 0.05	23.2 ± 3.7	7.24 ± 0.21	13.8 ± 2.4	68.2 ± 1.2
Pygmy	17	9.8 ± 0.2*	0.46 ± 0.01*	25.4 ± 2.2	1.08 ± 0.08*	19.2 ± 1.5	33.2 ± 2.3*

[a] Age: 90 days.

* $P < 0.01$, comparisons shown are between mice of the same sex.

sed by King (2) and supported by Ri- and Richmond (3), or whether it is due lack of other growth factors such as d hormones, or a lack of pulsatile re- of pituitary somatotrophic hormones, is ear at this time. Other possibilities may xist. For example, the immunoreactive nd PRL circulating in the plasma of y mice may have only weak biological y. There is growing evidence to suggest he preponderant isomers of GH and ound in the pituitary gland are prohor- s that undergo chemical transformation g or after secretion in order to manifest iological effects (12). We do not know enzymatic capabilities to catalyze such cal transformations are deficient or nt in the pygmy mouse. The failure of y mice to respond appreciably to the istration of GH (3) and PRL (Fig. 1) d from the pituitary raises this type of ility.

matically subnormal body growth rate presence of normal basal GH and PRL ntrations in the blood and the lack of rmal response to stimulation of PRL e by the pituitary gland make the y mouse an exceptionally interesting an- nodel for the study of mechanisms of nal regulation of growth.

mary. Basal levels of GH and PRL in rum and pituitary gland of normal (*Pg/* nd pygmy (*pg/pg*) (VY × C3H) F-1 l mice were measured by homologous mmunoassays. The serum concentra- of GH and PRL in pygmy mice at ximately 90 days of age were found to different from those in corresponding l controls. The pituitary concentrations two hormones, however, were substan-

E II. SERUM PROLACTIN CONCENTRATIONS OF MICE AFTER AN INJECTION OF PERPHENAZINE

nd ype	Hours after injection[a] 0	½	1
al	8 ± 1(8)	45 ± 2(8)	45 ± 2(7)
y	12 ± 1(6)	10 ± 2(6)	12 ± 1(5)
al	24 ± 7(7)	317 ± 32(6)	559 ± 39(8)
y	17 ± 3(7)	23 ± 2(6)	21 ± 2(6)

e of perphenazine = 1 μg/g BW. Values given n ± SEM. Numbers in parentheses indicate the of mice in each group.

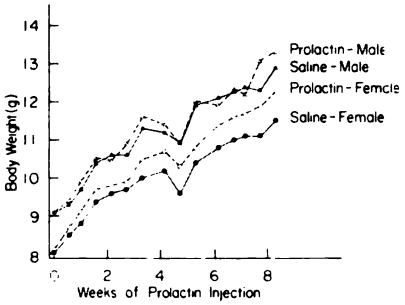

FIG. 1. Effect of daily administration of 100 μg ovine prolactin on body weight gain of pygmy (*pg/pg*) (VY/Wf × C3H/Wf) F-1 hybrid mice. 8–10 mice/group.

tially subnormal in pygmy mice. Perphenazine injection failed to stimulate PRL release by pygmy (*pg/pg*) pituitaries, in contrast to the marked increase in serum PRL levels of the normal *Pg/Pg* mice. Daily administration of 100 μg of ovine PRL for 8 weeks produced none or very little increase in the body weight of pygmy mice. These results suggest that the *pg* mutation induces a multifaceted change affecting the synthesis, release, and function of growth hormone and prolactin.

The ovine PRL used in these studies was supplied by the Pituitary Hormone Distribution Program of the National Institute of Arthritis, Metabolic, and Digestive Diseases.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 224-226 (1979)

In Vitro Inhibition of the Rate of Erythrocyte Sickling by RMI 11,071A and Its Possible Mechanism (40652)

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The importance of the interaction between the erythrocyte membrane and the abnormal hemoglobin aggregates in sickle cell anemia (SCA) has been well documented in recent years (1, 2). Though the genetic abnormality lies in the hemoglobin molecule, the functional lesion may lie in an abnormal membrane. The membrane of the sickle cell contains a much higher concentration of calcium (Ca) than that of the normal cell (3). It has also been shown that the ionophore A23187 will induce an influx of Ca, which produces bizarre shapes and stabilizes the sickled cell (4, 5). This stabilization will prevent the sickled cell from unsickling on reoxygenation similar but not identical to the irreversible sickled cell (ISC).

In a previous paper (6) we described a simple *in vitro* method for screening compounds for inhibition of the rate of sickling. We also described the activity of a compound that inhibited sickling rate and affected Ca content of the cells.

The purpose of this communication is to report the activity of RMI 11,071A, hexahydro-2-(α-methyl-*p*-phenoxybenzyl)-imino azepine hydrochloride, on the rate of sickling at 100 μg/ml of blood and its influence on Ca fluxes. RMI 11,071A is a member of a series of compounds, several of which have been found to inhibit platelet aggregation (7).

Materials and methods. Rate of sickling. The methods and apparatus for measuring sickling rate have been previously described (6). This procedure involves collecting blood from adult subjects who have SCA into 3.8% sodium citrate (1 part citrate to 9 parts of blood). Samples (3 ml) of the citrated blood were placed in serum bottles and cylinders of various gases (95% O_2 and 5% CO_2 for high oxygen tension or 2.5% O_2, 5% CO_2, and 92.5% N_2 for low oxygen tension) were connected through a manifold and flowmeter system to the blood bottles. The blood was first exposed to high O_2 tension to reduce the sickled cells to a minimum. Then either saline or RMI 11,071A solution was added and high O_2 tension maintained for 15 more min. A blood sample was then taken and fixed in 3% neutral formalin. The gas was then changed to the low O_2 tension. Blood samples were taken after 15, 30, 45, and 60 min and processed. Pictures of cells taken through Normarski interference optics were coded and at least 300 cells counted to determine percentage sickled cells. A cell was counted as a sickled cell if it had one or more points.

^{45}Ca flux. Blood was prepared as previously described except heparin (0.1 ml of 20 mg/ml for every 10 ml of whole blood) was used as the anticoagulant. Whole blood, 3.2 ml, was placed in the serum bottles, 0.4 ml of 50 μCi/ml of $^{45}CaCl_2$. (Final concentration of $^{45}CaCl_2$ was 5 μCi/ml of blood.) The bottle was placed in the apparatus and oxygenated for 30 min with 95% O_2, 5% CO_2. Samples were taken for analysis and deoxygenated for 30 min with 95% N_2, 5% CO_2 and again samples taken for analysis. The blood was then reoxygenated for 30 min and samples were taken for analysis.

Blood, 0.5 ml, was taken from each bottle through the rubber stopper with an 18-gauge needle into a 1-ml syringe. The samples were washed twice with 4.5 ml of N_2-saturated saline, then diluted to 1 ml with saline and the erythrocytes counted with a Coulter counter. One-half milliliter of the diluted cells was added to 4.5 ml of 6.7% TCA mixed, and centrifuged 1250*g* for 10 min at 4°. Two-tenths milliliter of the supernatant was counted in a scintillation counter. The radioactivity of each sample was compared to the total count added to the blood sample and is expressed as a percentage of ^{45}Ca dose/10^6 erythrocytes.

Results. Effects on sickling rate. The effect of RMI 11,071A at 100 μg/ml on sickling rate

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wn in Fig. 1. The data in this figure are
omposite of two experiments of 6 con-
and 6 tests each (total of 12 determina-
for each group). In experiment 1 inhi-
i of the sickling rate was 46.5% at 0–15
nd 43.3% at 15–30 min, for an inhibition
.1% for the 0- to 30-min period. In the
d experiment inhibition of the sickling
vas 30.1% at 0–15 min and 33.8% at 15–
in for a total inhibition for 0–30 min of
. The compound inhibited the rate of
ng for the first 30 min in both experi-
s. By 45 min of low O_2 tension the
itory effect had disappeared and the
ng rate of the test had increased enough
the number of sickled cells was ap-
:hing the control group by 60 min.

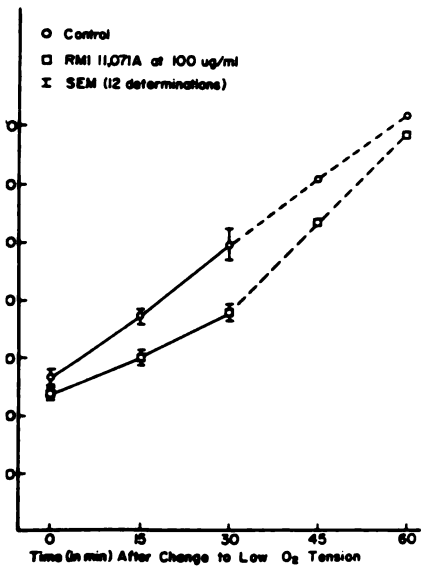
AI 11,071A at 100 μg/ml did not effect
pCO_2, or the oxygen dissociation curve.
average pH values (±SEM) with and
out compound were 7.324 ± 0.018 and
± 0.021 respectively; the pCO_2 values
and without compound were 39.2 ± 2.1
39.9 ± 1.4, respectively, and the P_{50}
s with and without compound were
± 0.55 and 32.95 ± 1.00, respectively.

ect on calcium fluxes. The results are shown in Table I. The amount of ^{45}Ca incorporated into the erythrocytes on deoxygenation was higher in the test group than in the controls. However, on reoxygenation the control group incorporated slightly more Ca (+6%), while the test groups lost a substantial amount of that previously incorporated (−20%).

Discussion. RMI 11,071A at 100 μg/ml inhibited the rate of sickling in an *in vitro* test system. This compound had no effect on pH, pCO_2, or the oxygen dissociation curve, but did affect Ca fluxes, results similar to those found for RMI 6792 reported in our previous paper (6). Since the erythrocytes from patients with SCA contain high levels of Ca, which increases as the cells are repeatedly deoxygenated and reoxygenated (sickled and unsickled) until they will no longer unsickle and become rigid and fragile, the functional lesion may be at or on the cell membrane. Dunn (8) reported that an increase in Ca influx caused an influx of Na and an efflux of K. He attributed the efflux of K to the cell-shape changes to echonocytic spherocytes. Glader and Nathan (9) discussed the relationship of Ca to Na–K shift and the ATP-energy connection but indicated that questions remain unanswered concerning the complete nature of the mechanism to the sickle-cell membrane lesion. The cell membrane in SCA has been shown to have an altered phosphorylation activity (10, 11).

Procaine and PABA (12) and zinc (13, 14) have been reported to inhibit Ca uptake and thereby improve cell filtration or decrease ISC count.

During the initial incubation of the sickled-cell/whole-blood samples with RMI 11,071A at high pO_2, the calcium pool may have decreased due to the effect of the compound on calcium efflux at high pO_2. Thus, the cell would contain less calcium, but more potassium ion (8). This loss of calcium would help explain the results, which showed an increased ^{45}Ca influx on deoxygenation even though the sickling rate was decreased over control. Even though the calcium pool size may be different between treated and untreated blood samples after the initial incubation, this difference would not cause the flux to change direction on reoxygenation. We were unable to measure total calcium



. 1. Effect of RMI 11,071A at 100 μg/ml on
g rate. Percentage sickled cells versus time after
to low oxygen tension (2.5%). Composite of two
nents with 6 determinations in each (total of 12
inations) ± SEM.

TABLE I. EFFECT OF RMI 11,071A AT 100 μg/ml ON CALCIUM INFLUX AND EFFLUX IN SICKLE CELL ERYTHROCYTES

Group	Percentage calcium ($45 \times 10^{-4}/10^6$ erythrocytes ± SEM)		
	Start	After deoxygenation for 30 min	After reoxygenation for 30 min
Control	322 ± 69	325 ± 66	345 ± 40
Change from previous value	—	+3	+20
RMI 11,071A at 100 μg/ml	389 ± 44	503 ± 87	403 ± 68
Change from previous value	—	+114*	−100*

* Significantly different from control.

content in these experiments due to breakdown of instrumentation.

Though the absolute calcium content was not measured, we propose that RMI 11,071A reduces the effect of Ca on the sickle cell system by improving the ability of the cell to decrease the Ca content upon reoxygenation. This may lower the K loss and reduce the rate of sickling initially on deoxygenation.

Summary. RMI 11,071A, one of a series of lactamimides, was found to inhibit the sickling rate in an *in vitro* system. This inhibition was not total or permanent; the sickling rate increased after 30 min at low O_2 tension and the number of sickled cells approached that of the controls by 60 min. There was no effect on pH, pCO_2, or the oxygen dissociation curve. ^{45}Ca fluxes were affected. Though influx of ^{45}Ca increased on deoxygenation, the compound caused a decrease in ^{45}Ca content on reoxygenation. This decrease was absent in the control group.

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OS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 227-228 (1979)

2,3,2-Tetramine—A Potent Cupruretic Agent (40653)

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treatment of Wilson's Disease (hepacular degeneration) became practical fective with the availability of D-penine, a chelator which is effective after dministration (1). However, the inciof hypersensitivity or toxic reactions to cillamine is high (2, 3). Although some ts can be desensitized, there is need for ɔnal therapeutic agents that can be betɛrated. The linear tetramine, triethylene ine (trien), or 2,2,2-tetramine,[2] has hown to be effective in patients with sed copper stores, and therefore, a useɛrnative to D-penicillamine (4–7).

in vitro interaction of Cu(II) with D-llamine (9) involves complex redox stry while the interaction of Cu(II) with ',2-tetramine is a simple issue of metaling agent equilibrium. The coordinaf the 2,2,2-tetramine to Cu(II) results in ned arrangement of three linked fivered chelate rings (8). This strain can eved by the central six-membered cheng present in the Cu(II) complex of the logous 2,3,2-tetramine with an increase formation constant (log K_1) from 20.2 9 (8). In addition to having a higher tion constant for the Cu(II) complex, rification of the 2,3,2-tetramine is simnce there are no potentially toxic isoformed as in the synthesis of trien (6,).

ause of these features, the 2,3,2-tetraor (3,7-diazanonane-1,9-diamine) was igated to determine its effectiveness *in* s a chelating agent for copper in rats following intravenous and oral administration.

Materials and methods. The 2,3,2-tetramine was synthesized by the method of Van Alpen (12), and converted to its tetrahydrochloride salt. Following recrystallization from an ethanol/dilute aqueous HCl mixture, the salt melted at 281–286° with decomposition (sealed cap.). Analysis calculated for $C_7H_{24}N_4Cl_4$: C, 27.47, H, 7.90; N, 18.30; Cl, 46.33. Found: C, 27.58, H, 7.95; N, 18.30; Cl, 46.14.

Rats (Sprague–Dawley) weighing 360–380 g were placed in restraining cages after a polyethylene tube (PE-190) was surgically placed in the bladder and connected to a fraction collector. The 2,3,2-tetramine (4.0 μmole/100 g body wt) was administered as its salt by continuous infusion for 3 hr via femoral vein or by gavage. Rats were normal or copper-loaded (13). The hourly urine volumes were measured and the urinary copper measured by atomic absorption spectrophotometry (14).

Results. The intravenous infusion of 2,3,2-tetramine (4.0 μmole/100 g body wt) resulted in a prompt cupruresis which was sustained during the infusion period—see Fig. 1. Copper excretion increased 70-fold and was at least 5-fold greater than the control period for 6 hr after the infusion was discontinued. Copper excretion following the infusion exceeded the control period during the 12 hr of observation.

Figure 2 shows the urinary copper excretion follows administration of 2,3,2-tetramine by gavage (4.0 μmole/100 g body wt). There was a delay in cupruresis but by 2 hr there was a sevenfold increase in copper-loaded rats. This cupruresis was sustained for the 12 hr of the study; copper excretion was at least twice the control values for 10 hr.

Discussion. Administration of the chelating agent, 2,3,2-tetramine, results in a cupruresis when given intravenously or orally. This cu-

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onvenient shorthand nomenclature has recently d in the chemical literature (8) whereby varear tetramines are characterized by the num- CH_2 units in each bridge. Thus, trien $H_2CH_2NHCH_2CH_2NHCH_2CH_2NH_2$) is a 2,2,2-ne.

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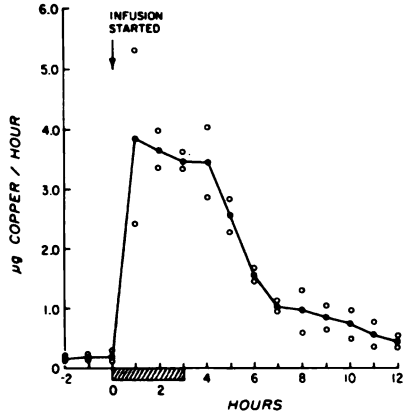

FIG. 1. Infusion of 2,3,2-tetramine, 4.0 μmole per 100 g body wt, started at 0 hr. Infusion continued for 3 hr (shaded area). Urinary copper values were obtained on two rats over a 14-hr period; individual values are designated with O and average by ●. The average values for the two rats are connected by the solid line.

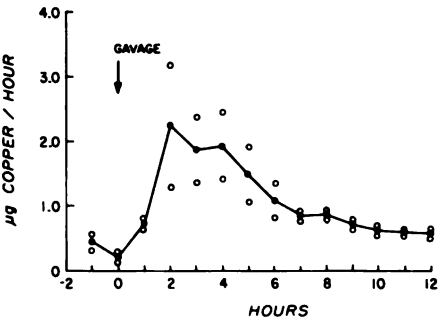

FIG. 2. The urinary excretion of copper following gavage with 2,3,2-tetramine. The dose, 4.0 μmole per 100 g body wt, was administered to two copper-loaded rats at 0 hr. Urinary copper values were obtained over a 14-hr period; individual values are designated with O and average by ●. The average values for the two rats are connected by the solid line.

pruresis was marked, particularly following intravenous administration, and lasted for several hours. This agent may prove to be an effective drug for those individuals requiring therapy for Wilson's disease or other diseases characterized by excessive copper stores, particularly in those individuals who show evidence of hypersensitivity or toxicity to D-penicillamine. Studies are in progress to determine effectiveness in comparison to D-penicillamine and trien.

Summary. The 2,3,2-tetramine is a potent cupruretic agent which may prove to be useful in management of disorders characterized by excessive copper stores.

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ıct of Retinoids and Estrogens on Testosterone-Induced Hyperplasia of Mouse Prostate Explants in Organ Culture (40654)[1]

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inoma of the prostate is one of the ; causes of death from cancer in men ıe age of 55. Treatment of human ic cancer includes surgery, chemother- ıd radiation therapy (1, 2). Although chemotherapeutic agents against ic lesions are being studied, estrogen ent (with or without orchiectomy) re- the principal therapy for human pros- ncer (1, 2). Several antiandrogens that prove useful for prostatic lesions have en proposed (1, 3–5).

noids have been shown to inhibit or carcinogen-induced epithelial lesions ıyperplasia and anaplasia) in mouse e organ cultures (6–10), and recently, e shown that all-*trans*-(β)-retinoic acid ed and reversed testosterone-induced lasia in mouse prostate organ culture ince testosterone has been linked with prostatic cancer and induces hyper- lesions of prostatic epithelium in organ (12, 13), this system seemed to provide erimental approach to determine if ds, estrone, and 17β-estradiol could influence testosterone-induced pros- yperplasia.

erials and methods. Organ cultures of prostate were prepared as previously ed (6, 7, 11). Ventral prostate from 8- veek-old C3H mice were excised and into 1.5 × 1.5-mm explants. The ex- were placed on lens paper supported inless-steel grids contained in 35-mm petri dishes. The cultures were incu- at 37° in CMRL 1066 medium supple- l with 10% fetal bovine or horse serum, per milliliter of penicillin and 100 μg/ ptomycin using a gas phase of 50% O_2 N_2 + 5% CO_2. The retinoids and ns were dissolved in dimethylsulfoxide (DMSO) at 4 mg/ml and were diluted in culture medium to obtain the desired concentrations. A final DMSO concentration of less than 0.2% in the culture medium was used in all experiments. DMSO at the corresponding concentration was also added to the control cultures. All experimental work was carried out in subdued light.

Two series of experiments were carried out. For the first series, 13-*cis*-retinoic acid or the estrogens at different concentrations were added simultaneously with testosterone hemisuccinate (Sigma Chemical Co.) to determine if the induction of hyperplasia could be prevented. In the second series of experiments in which the reversal of hyperplasia was determined, explants were first made hyperplastic by treatment with testosterone for 8 days followed by simultaneous treatment with testosterone and different concentrations of either a retinoid or an estrogen for an additional 96 hr.

The changes in the rates of proliferation caused by testosterone or a test compound were estimated by the Colcemid metaphase arrest technique (14). Colcemid (2 μg/ml) was added to each culture dish 4 hr before the explants were fixed in buffered formalin. The explants were processed for histology, serially sectioned, and stained with hematoxylin and eosin as previously described (6, 7, 11). The number of arrested metaphases in 1000–2000 cells per explant was counted and the mitotic index (MI) for each explant was estimated. From each explant, 2–3 sections (8–10 sections per group) were scanned and the MI ± SD (standard deviation) calculated for each group (4–6 explants). The significance of the difference between the control and experimental groups was determined by the Student's t test. A P value less than 0.05 was considered significant.

Since a certain degree of variability occurred in the values of mitotic indices in different experiments, each experiment in-

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cluded a series of untreated explants (untreated control group) and a series of explants treated with testosterone (testosterone control group) for each time period. All experiments were repeated and similar results were obtained. The data included in each table represent a single experiment.

Results. Testosterone-stimulated epithelial proliferation at 4 and 8 days after treatment, but when explants were incubated simultaneously with testosterone and 13-*cis*-retinoic acid, the induction of hyperplasia was prevented (Table I). For instance, at 4 days, the MI in the control explants was 98 ± 38, whereas the corresponding value in the testosterone-treated explants was 700 ± 148. The MI in explants that were simultaneously treated with testosterone (1.3×10^{-5} *M*) and different concentrations of 13-*cis*-retinoic acid were 114 ± 59 and 151 ± 71 at 1.7×10^{-5} and 3.3×10^{-6} *M*, respectively. 13-*cis*-Retinoic acid produced a similar mitotic inhibition at Day 8.

In contrast to 13-*cis*-retinoic acid, estrone or 17β-estradiol at similar concentrations produced no mitotic inhibitory effect on testosterone-induced hyperproliferation (Table II). Further, estrone or 17β-estradiol had no significant effect on the MI of the explants as compared to the untreated control group of explants (Table II).

Testosterone stimulated mitoses for at least 12 days (Table III). When explants were treated with testosterone for 8 days followed by simultaneous treatment with testosterone and a retinoid at 1.7×10^{-5}, 3.3×10^{-6}, and 6.6×10^{-7} *M* for an additional 96 hr, the hyperplasia was reversed. For example, at 12 days, the MI in the control explants was 53 ± 25, whereas in those treated with testosterone, the corresponding value was 272 ± 57. The MI in explants that were first treated with testosterone then with testosterone and different concentrations of 13-*cis*-retinoic acid for an additional 96 hr were 96 ± 57, 100 ± 58, and 74 ± 36 at 1.7×10^{-5}, 3.3×10^{-6}, and 6.6×10^{-7} *M* concentrations, respectively. Retinoids themselves, at these concentrations, had no effect on the MI of explants as compared to the MI of untreated control groups of explants (data not shown; see Ref. (6, 7)).

A similar treatment of hyperplastic cultures with estrone or 17β-estradiol, however, did not produce a reversal of hyperplastic lesions (Table IV). In fact, 17β-estradiol appeared to act synergistically with testosterone as mitotic stimulation was observed in the explants.

Discussion. Synthetic retinoids have potential value in the prophylaxis and treatment of epithelial lesions (15–17), and experimental evidence indicates that structural modifications of the retinoid molecule may enhance activity with a concomitant decrease in toxicity (18, 19). An effective side-chain modification is the 13-*cis*-isomer of a retinoid. In this study, 13-*cis*-retinoic acid can directly inhibit and reverse testosterone-induced hyperplasia in mouse prostate explants (Tables I and III). As a geometric isomer of all-*trans*-(β)-retinoic acid, 13-*cis*-retinoic acid is active in altering epithelial differentiation and in reversing benzo(α)pyrene-induced metaplasia (8, 18, 20). The 13-*cis*-isomer of retinoic acid is less toxic than all-*trans*-(β)-retinoic acid (18, 21, 22), reduces the incidence of carcinoma in hamsters treated with benzo(α)pyrene–ferric oxide (18), and re-

TABLE I. INHIBITION OF TESTOSTERONE HEMISUCCINATE-INDUCED HYPERPLASIA BY DIFFERENT CONCENTRATIONS OF 13-*cis*-RETINOIC ACID

Days after treatment	Control (MI ± SD)[a]	Testosterone 1.3×10^{-5} *M* (MI ± SD)	13-*cis*-Retinoic acid concentration[b] (MI ± SD)		
			1.7×10^{-5} *M*	3.3×10^{-6} *M*	6.6×10^{-7} *M*
4	98 ± 38	700 ± 148[c]	114 ± 59[d]	151 ± 71[d]	250 ± 112[d]
8	150 ± 38	542 ± 112[c]	134 ± 44[d]	138 ± 51[d]	493 ± 98

[a] The number of arrested metaphases per 1×10^5 cells during a 4-hr period of Colcemid treatment. SD = standard deviation.

[b] Explants were simultaneously treated with testosterone hemisuccinate and different concentrations of 13-*cis*-retinoic acid for 4 or 8 days.

[c] The mitotic stimulation was significant as compared to the corresponding control group ($P < 0.05$).

[d] The mitotic inhibition was significant as compared to the corresponding group that only received testosterone hemisuccinate ($P < 0.05$).

TABLE II. LACK OF INHIBITION OF TESTOSTERONE HEMISUCCINATE-INDUCED HYPERPLASIA BY DIFFERENT CONCENTRATIONS OF ESTRONE OR 17β-ESTRADIOL

Hormone	Days after treatment	Control (MI ± SD)[a]	Testosterone 1.3×10^{-5} M (MI ± SD)	Estrogen concentration[b] (MI ± SD)		
				1.8×10^{-5} M	3.7×10^{-6} M	7.4×10^{-7} M
Estrone	4	142 ± 70		113 ± 26	161 ± 43	—
	8	139 ± 31		158 ± 37	193 ± 68	177 ± 47
Estrone + Testosterone	4	80 ± 21	190 ± 58[c]	458 ± 156	480 ± 65	460 ± 84
	8	113 ± 33	1005 ± 162[c]	954 ± 213	614 ± 76	711 ± 307
17β-Estradiol	4	142 ± 70		121 ± 47	116 ± 35	102 ± 16
	8	139 ± 31		169 ± 56	229 ± 65	226 ± 70
17β-Estradiol + Testosterone	4	127 ± 30	471 ± 120[c]	547 ± 236	457 ± 154	353 ± 113
	8	164 ± 63	419 ± 165[c]	349 ± 128	455 ± 147	809 ± 189

[a] The number of arrested metaphases per 1×10^5 cells during a 4-hr period of Colcemid treatment. SD = standard deviation.

[b] Explants were treated with either estrone or 17β-estradiol or were simultaneously treated with estrone plus testosterone hemisuccinate or 17β-estradiol plus testosterone hemisuccinate for 4 or 8 days.

[c] The mitotic stimulation was significant as compared to the corresponding control group ($P < 0.05$).

TABLE III. REVERSAL OF TESTOSTERONE-INDUCED HYPERPLASIA BY DIFFERENT CONCENTRATIONS OF RETINOIDS

Retinoid	Days after treatment	Control (MI ± SD)[a]	Testosterone 1.3×10^{-5} M (MI ± SD)	Hours after adding retinoid	Retinoid concentration[b] (MI ± SD)		
					1.7×10^{-5} M	3.3×10^{-6} M	6.6×10^{-7} M
13-*cis* Retinoic acid	8	149 ± 49	480 ± 120[c]				
	12	53 ± 25	272 ± 57[c]	96	96 ± 57[d]	110 ± 58[d]	74 ± 36[d]
The 1-methoxyethylcyclopentyl analog of retinoic acid	8	251 ± 55	436 ± 91[c]				
	12	134 ± 52	366 ± 89[c]	96	—	71 ± 13[d]	151 ± 47[d]
The trimethylmethoxyphenyl analog of retinoic acid	8	146 ± 63	619 ± 40[c]				
	12	135 ± 42	430 ± 107[c]	96	103 ± 40[d]	113 ± 43[d]	152 ± 58[d]
The trimethylmethoxyphenyl analog of retinoic acid ethylamide	8	146 ± 63	619 ± 140[c]				
	12	135 ± 42	430 ± 107[c]	96	122 ± 51[d]	157 ± 59[d]	101 ± 34[d]
The trimethylmethoxyphenyl analog of retinoic acid ethyl ester	8	251 ± 53	436 ± 91[c]				
	12	134 ± 52	366 ± 89[c]	96	152 ± 78[d]	82 ± 43[d]	275 ± 87

[a] The number of arrested metaphases per 1×10^5 cells during a 4-hr period of Colcemid treatment. SD = standard deviation.

[b] Explants were treated with testosterone hemisuccinate for 8 days followed by simultaneous treatment with testosterone hemisuccinate and different concentrations of retinoids for an additional 4 days.

[c] The mitotic stimulation ws significant as compared to the corresponding control group ($P < 0.05$).

[d] The mitotic inhibition was significant as compared to the corresponding group that received only testosterone hemisuccinate ($P < 0.05$).

duces the incidence and extent of bladder cancer in rats induced with *N*-methyl-*N*-nitrosourea (23) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (24). Peck and Yoder (15) have recently reported that in the treatment of patients with keratinizing dermatosis, 13-*cis*-retinoic acid may be more effective and less toxic than all-*trans*-retinoic acid.

The four retinoids (Chart 1) in which the β-ionone ring of all-*trans*-(β)-retinoic acid was replaced with a five- or six-carbon ring were active in reversing testosterone-induced hyperplasia in mouse prostate explants (Table III). Two of these retinoids were trimethylmethoxyphenyl analogs also modified at the terminal carboxyl group of the side chain (the ethylamide and the ethyl ester, Table III). Other reports have also indicated that the unaltered β-ionone ring of the retinoid molecule is not necessary for altering epithelial differentiation (18–20, 25) or for reversing hyperplasia of mouse prostate explants induced by *N*-methyl-nitro-*N*-nitrosoguanidine (7) or 3-methylcholanthrene (9,

10). The ethyl ester and ethylamide analogs of the trimethylmethoxyphenyl retinoids have also been reported to be active in reversing keratinization (18–20, 25), increasing RNA in epidermal cell cultures (18, 19), and in promoting the lysis of the extracellular matrix of cartilage in organ culture (9, 10).

Specific structural alterations of the retinoid molecule can affect different biological activities. Thus while the cyclopentenyl retinoid is more effective than all-*trans*-(β)-retinoic acid in modulating epithelial differentiation (20, 25) and highly active in inhibiting and reversing the effects of carcinogens on mouse prostatic epithelium *in vitro* (7–10), it is virtually devoid of vitamin A growth-promoting activity (26) and is highly effective in promoting the lysis of cartilage extracellular matrix in organ culture (26).

Estrone or 17β-estradiol had no effect on the MI of mouse prostate explants in this system (Table II). The effect of estrogens on prostatic epithelium may be dependent on the age of the explanted tissue. For instance in organ cultures of prostates from 6-week-old mice, estrone- and stilbesterol-induced

TABLE IV. LACK OF REVERSAL OF TESTOSTERONE HEMISUCCINATE-INDUCED HYPERPLASIA BY DIFFERENT CONCENTRATIONS OF ESTRONE OR 17β-ESTRADIOL

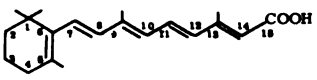
Estrogen	Days after treatment	Control (MI ± SD)[a]	Testosterone 1.3×10^{-5} *M* (MI ± SD)	Hours after adding estrogen	Estrogen concentration[b] (MI ± SD)		
					1.8×10^{-5} *M*	3.7×10^{-6} *M*	7.4×10^{-7} *M*
Estrone	8	118 ± 57	409 ± 149[c]				
	12	143 ± 49	513 ± 113[c]	96	782 ± 143	960 ± 134	610 ± 161
17β-Estradiol	8	119 ± 33	413 ± 141[c]				
	12	146 ± 13	594 ± 239[c]	96	1215 ± 161	1669 ± 578	1687 ± 458

[a] The number of arrested metaphases per 1×10^5 cells during a 4-hr period of Colcemid treatment. SD = standard deviation.

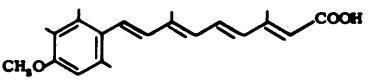
[b] Explants were treated with testosterone hemisuccinate for 8 days followed by simultaneous treatment with testosterone hemisuccinate and different concentrations of estrone or 17β-estradiol for an additional 4 days.

[c] The mitotic stimulation was significant as compared to the corresponding control group ($P < 0.05$).

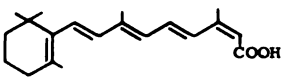
Structure of Retinoids



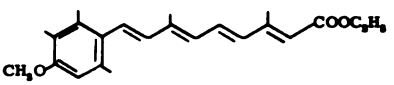
All-trans-retinoic acid



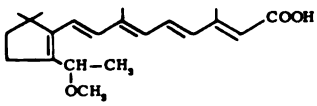
Trimethylmethoxyphenyl (TMMP) Analog of Retinoic Acid



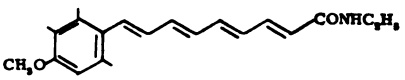
13-cis-Retinoic Acid



Trimethylmethoxyphenyl Analog of Retinoic Acid Ethyl Ester



The 1-Methoxyethyl Cyclopentenyl Analog of Retinoic Acid



Trimethylmethoxyphenyl Analog of Retinoic Acid Ethyl Amide

The retinoids were obtained from Hoffmann-La Roche, Inc. (Nutley, NJ and Basal, Switzerland) through the Lung Cancer Segment, NCI, Bethesda, MD.

asia of the alveolar epithelium (12, e, in prostate explants from 5- to 6- old mice, estrone produced atrophy rosis of the epithelium (12). The fail- estrogens to stimulate the explants from 8- to 10-week-old C3H mice e due to differences in susceptibility the mice used in the study and those other investigators or to the influence hormones, presumably present in the sed to supplement the medium. How- e prostate explants were evidently nsitive to stimulation with testoster- iisuccinate (1.3×10^{-5} *M*, Tables I-

ata show that estrogens failed to in- reverse testosterone-induced hyper- mouse prostate explants (Tables II). In agreement with these results, orts have indicated that *in vivo* 17β- had no significant effect on testos- timulated DNA synthesis in the pros- astrated rats (2, 28). Estrogens, how- use remission of prostatic lesions in 29). The mechanism by which estro- se remission of these lesions has not npletely defined. Estrogens may in- vith the metabolism of testosterone. mple, Farnsworth (30) showed that adiol or estrone inhibited the conver- testosterone to dihydrotestosterone n homogenates of prostate obtained ients with benign prostatic hyperpla- *et al.* (28) showed that 17β-estradiol 5α-reductase activity in the prostate rats, although no effect on the en- tivity was observed in the prostate of rats injected with testosterone and adiol. Estrogens are believed to influ- testosterone titers indirectly via the gonad axis. Our results showing that s had no effect on testosterone-in- perplasia in mouse prostate explants II and IV) suggest that estrogens do e any direct effect on testosterone ism in this system and therefore sup- view that an estrogenic effect in the n of prostatic lesions in man may the pituitary–gonad axis. In this re- der *et al.* (31) have suggested that s may decrease testicular testosterone on by inhibiting pituitary gonadotro- duction. This is probably true since estrogens inhibit prostatic growth of the intact animal by reducing serum testosterone levels, whereas they do not block testosterone-induced growth in castrated rats (32).

Recently (33) Burns *et al.* showed that estradiol (5×10^{-9} *M*) inhibited the binding of DHT in the cytosol preparations of the seminal vesicle of guinea pig while diethylstilbestrol (5×10^{-9} *M*) had no apparent effect on this binding, although both estrogens caused regression of seminal vesicles when injected in the intact animal. These authors thus suggested that at least in the case of seminal vesicles, estradiol, in addition to its influence via the pituitary–gonad axis, may also have a direct effect at the tissue level (34). It should, however, be pointed out that in the case of prostate cytosol preparations, estradiol at significantly higher concentrations (10^{-6} *M*) had no effect on DHT binding (34, 35).

In contrast to estrogens, retinoids can directly inhibit and reverse testosterone-induced lesions in the mouse prostate explants. The mechanism by which retinoids cause these antiandrogenic effects is also not known, although several possibilities can be suggested. For instance, it may act by blocking the uptake of testosterone by competing with the hormone at the appropriate sites. Recently, B. P. Sani (personal communication) showed that testosterone does not compete with all-*trans*-retinoic acid for binding with retinoic acid-binding protein of chick embryonic skin, thus suggesting that testosterone and all-*trans*-retinoic acid bind independently to their receptor proteins. However, it is possible that the 13-*cis*-retinoic acid–protein complex inhibits the binding between DHT–protein complex and nuclear protein. Fang and Liao (36) have demonstrated that cyproterone acetate, an antiandrogen, inhibited the transport of labeled DHT from the prostatic cytoplasm to the nucleus, and these results were confirmed by other investigators (37–39).

Summary. Testosterone produced epithelial hyperplasia in mouse prostate explants in organ culture. Simultaneous treatment of explants with testosterone and 13-*cis*-retinoic acid inhibited the induction of hyperplasia. When explants were first made hyperplastic by treatment with testosterone for 8 days and

followed by simultaneous treatment with testosterone and a retinoid for an additional 96 hr, the following retinoids reversed the hyperplasia: 13-*cis*-retinoic acid, the 1-methoxyethylcyclopentenyl analog of retinoic acid, the trimethylmethoxyphenyl analog of retinoic acid, the trimethylphenyl analog of retinoic acid ethylamide, and the trimethylmethoxyphenyl analog of retinoic acid ethyl ester. The data indicate that these retinoids prevent androgen-induced hyperplasia in prostatic tissue in this system by a direct influence on the tissue.

Since estrone or 17β-estradiol had no effect on testosterone-induced hyperplasia in the prostate explants, it is concluded that these estrogens may act by way of an indirect mechanism (pituitary–tesis axis) in preventing androgen-induced hyperplasia of the prostate.

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Experimental HSV Latency Using Phosphonoacetic Acid (40655)[1]

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viruses characteristically produce
/ith recurrent manifestations. Dur-
ymptomatic period, between initial
with herpes simplex virus (HSV)
rent manifestation, it is hypothe-
the virus is maintained in a "latent"
2). The cell–virus interactions in-
latency and reactivation have not
ied extensively due to the lack of
te *in vitro* models. Some HSV per-
nd latency models developed thus
e the addition of herpes virus-spe-
odies to the growth medium of
cted cells (3–5), drastic alterations
tion temperature of HSV-infected
), and use of cells that are semiper-
r HSV replication (8, 9). Recently,
rabinoside (ara-C), an inhibitor of
thesis, was utilized by O'Neill and
s to inhibit the replication of HSV
SV-2) over a 7-day period (10, 11).
of HSV replication by ara-C dur-
riod not only produced an interval
nfectious virus was undetectable,
od of 5–11 days after the removal
ibitor was required for the reap-
of infectious virus. Infectious virus
undetectable when infected cul-
: shifted from 37 to 40° at the time
emoval and were maintained at this
re. If, however, the infected cul-
tained at 40° were again placed at
tious virus reappeared 11–16 days
erature shift down (10).

entions were to study some of the
nts for establishment of latency by
ng whether inhibition of virus DNA
was sufficient to produce the latent
hether inhibition of cellular DNA synthesis was required. With this goal in mind, we selected phosphonoacetic acid (PAA), a preferential inhibitor of HSV DNA synthesis, for our latency studies. HSV previously has been shown to be sensitive to PAA inhibition (12, 13) at concentrations as low as 20–40 μg of PAA/ml when low multiplicities of infection (MOI) are used. This paper describes the successful induction of a latent period in cultured human cells using PAA.

Materials and methods. Experiments were performed on monolayers of human embryo lung (HEL) cells grown to confluence in Falcon T-25 flasks in Dulbecco's medium supplemented with 10% fetal calf serum (FCS; Flow Laboratories, Rockville, Md.), 0.03% glutamine, 0.075% sodium bicarbonate, 25 U penicillin/ml, 25 μg streptomycin/ml, and 100 μg kanamycin/ml. Maintenance medium contained 2% FCS, 0.15% sodium bicarbonate, and additional supplements similar to those in the growth medium.

Flow 5000 cells (Flow Laboratories) were passaged in T-75 flasks in modified Eagle's minimum essential medium supplemented with 10% FCS, 10% tryptose phosphate broth, 0.075% sodium bicarbonate, 100 U penicillin/ml, and 100 μg streptomycin/ml. Stocks of HSV-2 (strain 186) were grown in Flow 5000 cells. Confluent monolayers were infected at an MOI of 0.01 plaque-forming unit (PFU)/cell and infected cells were harvested when 90% of the cells showed cytopathic effects (CPE). Virus was obtained by freezing and thawing infected cells three times and eliminating cell debris by centrifugation at 800 *g*. All virus stocks were maintained at −70° and titered on primary rabbit kidney (PRK) cells overlaid with 0.5% methylcellulose (14). PAA (ICN Pharmaceuticals, Plainview, N.Y.) and the disodium salt of phosphonoacetate hydrate (Abbott Laboratories, Ill.) were used as DNA synthesis inhibitors. Latency experiments were carried out as previously described (10) with the following modifications:

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Cancer Institute.
it of a fellowship from the Ford Foundation
an Cancer Society Institutional Research
9.

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PAA was substituted for ara-C during drug treatment and virus titers were determined by plaque formation on PRK cells overlaid with 0.5% methylcellulose.

Results. When HEL cells were pretreated with 100 μg/ml of PAA, virus replication was delayed, but not inhibited, over a 7-day period demonstrating that the concentration of PAA used was not sufficient to inhibit HSV-2. Low levels of resistant virus (0.07%) present in unselected virus stocks were responsible for the virus replication observed in the presence of PAA. When HEL cells were infected in the presence of 100 μg PAA/ml for 3 or 5 days (PAA replaced daily), and the lysates were subjected to plaque titration in the presence and absence of PAA (100 μg/ml), the level of PAA-resistant virus present was 18–28% representing a 250- to 400-fold increase in the amount of resistant virus present in the original HSV-2 stock.

Drug dose studies were carried out to determine the concentration of PAA required to inhibit replication of low levels of PAA-resistant virus within original stocks. Results showed that a minimum of 200 μg PAA/ml was required to reduce infectious HSV-2 below detectable levels for 7 days.

We have shown previously that 25 μg/ml of ara-C will produce a latent period in HEL cells infected with HSV-2 strain 186 (15) similar to that observed with HSV-2 strain 316-D (10, 11). Although results were similar, the time of virus reappearance after ara-C removal differed in our system. When cultures were maintained at 37°, virus reappeared 4–30 days after ara-C removal. O'Neill and co-workers (10, 11), however, reported the reappearance of infectious virus 5–11 days after reversal of ara-C treatment. In addition, when cells were maintained at 40° and then shifted down to 37°, a shorter period (5 days after shift down) than that previously reported (11–16 days after shift down (10)) was observed.

To induce latency, twice the minimum concentration of PAA required for inhibition of HSV-2 (186) was used. Two separate experiments produced results similar to those observed with ara-C treatment. HEL cells pretreated with PAA (400 μg/ml) and infected with HSV-2 (186) were examined for the presence of virus (Fig. 1). Infectious virus was undetectable in the presence of the drug and did not reappear until 12 days after PAA removal (Day 19 p.i.) whereupon virus reappeared rapidly and reached high titer. The mean time of reactivation (18.5 days p.i.) was longer than that observed using ara-C-treated cultures (14.5 days p.i.). The absence of detectable amounts of infectious virus was extended by increasing the incubation temperature from 39.5 to 40° at the time of PAA removal (Fig. 2). The results were similar to the latency periods produced by ara-C.

Our HSV latency model used 400 μg/ml of PAA. This PAA concentration inhibited both HSV and cell DNA synthesis, yet produced little, if any, detrimental effect on confluent monolayers of HEL cells.

Discussion. Using PAA to differentiate between the inhibition of virus and cell DNA synthesis required for the establishment of latency was not possible since concentrations of PAA sufficient to prevent HSV-2 replication over a 7-day period inhibited cell as well as virus DNA synthesis. The mechanism by which PAA inhibits DNA synthesis has been well established (16–18); PAA preferentially inhibits herpes virus DNA polymerase. In contrast, the mechanism of DNA synthesis inhibition by ara-C is not well understood (19). We were able to demonstrate that PAA

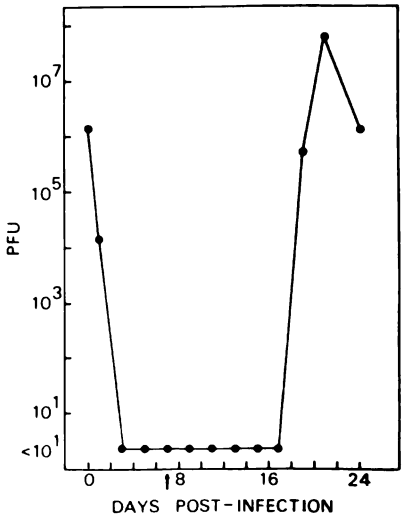
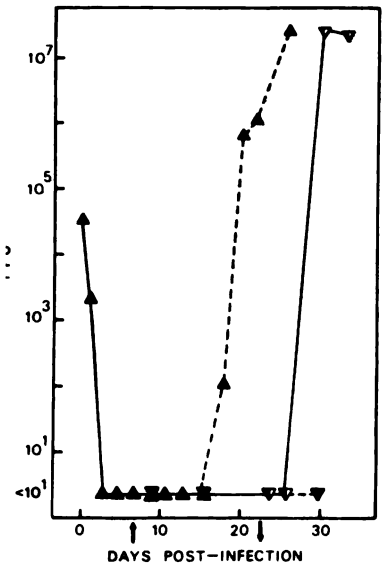


FIG. 1. Induction of HSV latency with PAA treatment (●). Cells were pretreated, infected, and treated with PAA (400 μg/ml) as described in Materials and Methods.



2. Extension of the latency period by alteration ation temperature to 40° at the time of drug Cells were treated in a manner similar to that hort-term latency experiments (Fig. 1) with the ı of alteration of incubation temperature to 40° .i. Control cells were maintained at 37° during PAA treatment (▲) while parallel cultures were ɔ 40° and maintained at 40° throughout the :nt (▼). A group of infected cultures shifted to lays p.i. was subsequently shifted to 37° 23 days

bitor of defined action, was capable of ng the appropriate conditions for the :hment of HSV-2 latency *in vitro*. In n, PAA appeared to be as effective as n inducing a period in which infectious undetectable. Studies in progress will t to characterize this period by exam- ıe expression of virus products and the the virus during this latent period.

mary. Treatment of herpes simplex vi- e 2 (HSV-2)-infected human embryo :lls with phosphonoacetic acid (PAA) ncentration of 100 μg/ml did not in- ıe replication of HSV-2 over a 7-day due to replication of low levels of :sistant virus present in original virus Dose–response curves demonstrated minimum of 200 μg of PAA/ml was required to inhibit HSV-2 over this time interval. At twice the minimum concentration of PAA required, it was possible to induce a period during which infectious virus was undetectable after reversal of drug treatment. This latent period was longer than that observed previously using cytosine arabinoside, and was extended by increasing the incubation temperature to 40° at the time of drug removal.

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Influence of Environmental pH on the Preservation and Inactivation of Herpes Simplex Virus (40656)

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Successful primary isolation of a virus from clinical specimens is enhanced by inoculating cell cultures as soon as possible. During the period between sample collection and culture inoculation, it has been recommended that the clinical specimen be refrigerated or frozen to preclude inactivation and maintain virus viability (1–3). The use of transport media has been proposed for clinical specimens that are delayed in transit to the laboratory, especially if they are stored at ambient temperatures. Although herpes simplex virus (HSV) is reputed for its lability (1), recovery of this virus from clinical specimens is facilitated by utilizing a transport medium (4–6). Thus, these media apparently stabilize the HSV by retarding inactivation and thereby promote the successful recovery of virus.

Inactivation of virus in clinical samples has been attributed to chemical substances in the specimen, e.g., enzymes and/or to the conditions of sample storage and handling (2). Temperature as an effector of the inactivation of HSV, has been the subject of numerous investigations (7). However, the facility and the kinetics of HSV inactivation at a given temperature has been shown to be modulated by the composition and pH of the medium in which the virus is suspended (8–10). This study was performed to characterize further the concerted effects of environmental pH and incubation temperature as a means of specifically promoting the inactivation or preservation of the replicative capability of HSV.

Materials and methods. The virus employed in this study was isolated from lesions associated with a recurrent infection of the volar surface of the hand. *In vitro* cultivation of this HSV isolate was limited to five passages in an attempt to minimize the selection of or elimination of virus subpopulations as previously suggested (11). Antiserum neutralization, plaque diameter, and pock size (12) all indicated the virus was an HSV type 2 (designated Strain SJ).

Stocks of HSV ($>10^8$ plaque-forming units (pfu) per milliter) were prepared by chemical concentration of virus from infected cell sonicates, using polyethylene glycol (13). The concentrated virus was diluted 10^{-2} into tissue culture medium containing 5% newborn calf serum and antibiotics, and supplemented with 0.025 *M* *N*-2 hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (hepes buffer (10)). The pH of the medium, adjusted to pH 6.3, 7.0, or 7.8 immediately prior to use, was unaltered as a result of the addition of virus. The HSV samples were incubated at 4, 36, or 40°. At select times, an aliquot of each sample was removed, diluted in Hanks' balanced salt solution (14), pH 6.6, and then plated on monolayers of rabbit skin cells. Following a 2-hr adsorption period at room temperature, the cultures received tissue culture medium and were incubated at 36° for 3 days. Plaques were visualized by crystal violet staining of the cell sheet as previously described (12). On occasion an HSV sample was stored at −85° prior to assay for pfu or a sample had to be reassayed for pfu content. In these instances, all three samples for that time point were treated in an identical manner. Preliminary observations indicated there was no difference in the stability of HSV suspended in tissue culture medium adjusted to pH 6.3, 7.0, or 7.8 when stored at −85°.

Results. The relationships between the environmental pH, the incubation temperature and the inactivation of this HSV-2 strain were examined. The data indicate the following: At each temperature (4, 36, and 40°) as the pH of the medium was increased from 6.3 to 7.0 to 7.8, the HSV surviving fraction decreased. In addition, as the incubation temperature was increased the HSV surviving fraction decreased at each pH tested (Figs. 1A–C). Thus, the combination of elevated temperature and an alkaline pH promoted HSV inactivation, while the combination of lower temperature and acidic pH promoted HSV survival.

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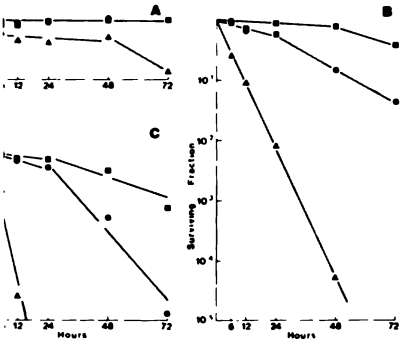

1. A stock HSV-2 (Strain SJ) preparation was 0^{-2} into tissue culture medium adjusted to pH .0 (●), or 7.8 (▲) and incubated at 4 (panel A), l B), or 40° (panel C). The pfu content in each /as determined at the times indicated. The 0-hr ıtent for all samples was approximately 6×10^5

reversibility of the pH-associated in- on effects was also examined. An HSV ıtion, diluted into medium adjusted to , was incubated at 36° for 24 hr. At ıe, the pH of the medium was adjusted nd the sample was reincubated at 36° ıdditional 48 hr. Virus survival during hr incubation period was determined ying the HSV pfu. The data indicate : HSV inactivation at pH 7.8 is irre- : and unresponsive to the subsequent 'e of the virus to an environment ›romotes virus stability (pH 6.3). The rther reflect the influence of environ- pH (7.8 vs 6.3) on the rate of HSV ıtion (Fig. 2).

ıssion. The results of previous studies : thermal inactivation of HSV is influ- ›y three factors: the temperature, the ition, and the pH of the medium in the virus is suspended (8–10). The presented in this report provide evi- eflecting upon the antagonistic or pro- .ic effects of pH on the inactivation of plicative capability suspended in tis- ure medium and incubated at various ıtures. The calculated postlag period :s at 36°, pH 6.3, 7.0, and 7.8, of this passage recent clinical HSV isolate ith the values obtained for the plaque- . laboratory strains of HSV types 1 10). In addition, the influence of pH preservation or inactivation of HSV has been shown to include 4 and 40°. Thus, these observations permit the selection of an appropriate environment to promote or retard the inactivation of HSV, commensurate with the desire of the investigator.

Preservation of HSV replication potential is requisite for primary virus isolation from clinical specimens. Considering delays associated with transporting samples to the clinical laboratory, a number of investigators have examined the utilization of transport media to enhance HSV recovery when clinical samples are held at ambient temperature for varying periods of time (4–6). Most of the media employed were modified bacteriologic transport media which have a particulate consistency due to the presence of charcoal, agar, agarose, and/or DEAE-dextran. The transport medium pH (7.6) was listed for only one of the media tested, in only one report (4). In addition, one study compared Stuarts medium with a virus transport medium (VTM) which consisted of tissue culture medium containing 10% fetal calf serum (5). In this latter instance the recovery of HSV from clinical specimens held in VTM for 1 to 2 days prior to processing was less than from specimens processed on the day the swabs were taken. This decrease in the efficiency of primary HSV isolation may be a reflection of HSV inactivation at pH >7.0, although the

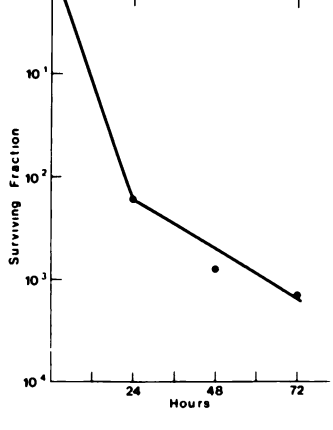

FIG. 2. A stock HSV-2 preparation was diluted 10^{-2} into tissue culture medium adjusted to pH 7.8 and incubated at 36° for 24 hr. The pH of the medium was then adjusted to 6.3. The pfu content was determined at the times indicated. The 0-hr virus content was 3×10^6 pfu/ml.

pH of the VTM was not specifically listed (5). Considering the influence of the composition of the suspending medium on the inactivation of HSV (8–10), a direct comparison of the previous studies with the data in this report is not meaningful. However, the data presented here clearly indicate the lack of HSV inactivation at 4° when virus is suspended in tissue culture medium adjusted to pH 6.3 or 7.0. The utility of this finding as a means of enhancing the primary isolation of HSV from clinical specimens is anticipated.

The ability of a virus to induce the oncogenic transformation of a cell is dependent upon survival of the cell following infection by the virus. In the case of HSV, this has been accomplished by infecting cells with virus previously inactivated by ultraviolet light (15), photodynamic dye (16), or by incubating HSV-infected cells under environmental conditions which preclude virus replication and cytotoxicity (17). A recent report also documented the ability of heat-inactivated (56°) HSV to transform mammalian cells to a thymidine kinase-positive phenotype (18). These observations imply that despite a loss of HSV replicative capability, at least a portion of the thermally inactivated HSV was capable of infecting these cells. Studies in our laboratory on the physical integrity and adsorptive capability of thermally inactivated HSV support this support notion (19). Thus, the observations presented here provide specific means to promote the thermal inactivation of HSV. The utility of this procedure, as a means of producing a virus population which could be employed to examine the transformation and/or oncogenic potential of HSV, remains to be investigated.

Summary. Incubation temperature and environmental pH were evaluated as independent and interdependent effectors of HSV inactivation. Kinetic studies at 4, 36, and 40° showed HSV inactivation was increased as the pH of the suspending medium was increased from 6.3 to 7.0 to 7.8. Conversely, the rate of HSV inactivation was antagonized and significantly reduced at each of these temperatures by adjusting the pH of the suspending culture medium to an acidic level. The utility of these observations as a means of enhancing the successful recovery of HSV from clinical specimens warrants consideration.

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Purification of Neonatal Rat Pancreatic Monolayer Cultures for Endocrine Cells (40657)[1]

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Although monolayer cell cultures of the pancreas have been successfully established by several investigators and used in studies of islet hormone release, islet cell morphology, and islet cell replication, fibroblastoid cells which are established in culture along with the endocrine cells rapidly proliferate and eventually overgrow the endocrine cells so as to inhibit their further development. Selective attachment procedures (1), chemically defined medium (2), cystine-free medium (3), addition of sodium ethylmercurithiosalicylate (4, 5), and selective enzymic detachment of endocrine cells from monolayers (6) have all been reported to enrich pancreatic monolayer cultures for endocrine cells. The present studies were undertaken to assess the effectiveness of two additional approaches to this problem.

Medium in which D-valine has been substituted for L-valine has been reported to selectively inhibit fibroblast proliferation while allowing growth of epithelial cells (7). The incorporation into DNA of bromodeoxyuridine added to nutritionally deficient medium is lethal to cells able to proliferate under these conditions, whereas those cells unable to synthesize DNA under such conditions are not destroyed (8). Both of these methods were evaluated for their ability to curtail fibroblast proliferation in pancreatic monolayer cultures.

Materials and Methods. Materials. Trypsin (1:250) was from Difco; type IV collagenase from Worthington; plastic tissue culture dishes from Falcon or Lux; NCTC-135, Medium 199, nonessential amino acid mixture, individual amino acid concentrates, and fetal bovine serum from GIBCO; gentamicin and Eagle's MEM from Microbiological Associates; bromodeoxyuridine (BUdR) from Calbiochem; and D-valine from Sigma.

Preparation of cultures. Pancreases were removed from 3- to 5-day-old Wistar rats, washed at room temperature in Ca- and Mg-free phosphate-buffered saline (PBS) for 5 min, and dissociated by sequential 10-min incubations at 37° in Ca- and Mg-free PBS containing 0.5% trypsin and 0.02% collagenase (1). Both wash and dissociation were aided by stirring with a magnetic stirrer. For each experiment, one pool of neonatal rat pancreases was enzymatically dispersed in this manner. Dissociated cells were collected, washed with medium, and dispensed into 100-mm diameter plastic tissue culture dishes so that each dish received cells from approximately four pancreases. The culture medium usually employed in our laboratory for establishment of these cultures has consisted of (v/v): 45% NCTC-135, 45% Medium 199, and 10% heat-inactivated fetal bovine serum (9–13). This medium is supplemented with glucose (16.5 m*M*) and gentamicin (50 μg/ml). However, in the studies reported here and shown in Tables I and III, culture medium consisted of Eagle's MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, nonessential amino acids (14), Earle's salts (15), gentamicin (50 μg/ml), and glucose (16.5 m*M*). Following incubation for 14–16 hr at 37° in a humidified atmosphere of 95% air and 5% CO_2, the cells which were unattached to the culture dishes were collected, the dishes gently rinsed with medium, and the rinses added to the collected cells. Following centrifugation, equal aliquots of cells were dispensed into 60-mm diameter plastic tissue culture dishes so that each dish contained cells from approximately one pancreas. However, results from one experiment may not be similar to those from another experiment since different pools of neonatal rat pancreas

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TABLE I. DNA CONTENT, INSULIN SECRETION, AND GLUCAGON SECRETION BY TREATED AND CONTROL CULTURES

Day of culture	Day after start of culture treatment[a]	DNA (μg/culture ± SE) (n)			Insulin (μU/ml ± SE) (n)			Glucagon (pg/ml ± SE) (n)		
		Control	D-Val	BUdR	Control	D-Val	BUdR	Control	D-Val	BUdR
6	4	5.08 ± 0.11 (3)	0.41 ± 0.02 (4) ***[b]	0.56 ± 0.05 (4) ***	1280 ± 24 (3)	1265 ± 17 (4) NS[b]	1200 ± 140 (4) NS	1673 ± 59 (3)	975 ± 27 (4) ***	1240 ± 37 (4) ***
9	7	11.28 ± 0.36 (4)	0.34 ± 0.14 (3) ***	2.16 ± 0.03 (4) ***	1698 ± 63 (4)	1083 ± 17 (3) ***	1340 ± 37 (4) ***	1300 ± 38 (4)	693 ± 138 (3) ***	1365 ± 38 (4) NS

[a] BUdR (10^{-5} M) and D-valine (92 mg/liter) were started on culture Day 2 (culture treatment Day 0). D-Valine was continued throughout the treatment period, but BUdR was added only for the first 24 hr, following which control medium was added. Control medium was Eagle's MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, nonessential amino acids, gentamicin (50 μg/ml), and glucose (16.5 mM). D-Val medium was similar to control medium except D-valine was substituted for L-valine, and fetal bovine serum was dialyzed. BUdR medium was also similar to control except BUdR (10^{-5} M) was added for the first 24 hr (until culture treatment Day 1).

[b] When compared to control, $P < 0.001$ (***). P = not significant (NS).

were used and the number of cells dispersed to each culture dish could differ between experiments. Tables I, II, and III give further experimental details for separate experiments.

Assays. Immunoreactive insulin (16), immunoreactive glucagon (17), and DNA (18) were measured using methods and materials described previously. Data in Table I were examined by analysis of variance using orthogonal contrasts (19), and by Newman–Keul's multiple comparisons procedure (20). Data in Tables II and III were examined by one-way analysis of variance and the Newman–Keul's procedure.

Results. When pancreatic monolayer cultures were maintained in control medium for 9 days, both insulin and glucagon secretion remained quite constant whereas the DNA content of the cultures increased substantially (Table I). It was apparent from examination of cultures in a phase-contrast microscope that this increase in DNA content was due almost entirely to fibroblast proliferation.

When either D-valine-substituted medium or BUdR treatment were used, cell proliferation was markedly curtailed as indicated in Table I by the significant reduction in DNA content on culture Days 6 (8% and 11% of control) and 9 (3% and 19% of control). On the other hand, both insulin and glucagon release were not affected to a comparable degree. For example, on culture Day 6, DNA was reduced to 8 and 11% of control with D-valine medium and with BUdR treatment, respectively, whereas glucagon release was reduced to only 58 and 74% of control for D-valine and BUdR, respectively, and insulin release was virtually unchanged from control. Furthermore, when cultures were maintained in D-valine medium, culture growth remained curtailed as DNA content remained constant, but both insulin ($P < 0.01$) and glucagon ($P < 0.001$) release were reduced further. On the other hand, following BUdR treatment for 24 hr cultures recovered their proliferative capacity as DNA increased fourfold between culture Days 6 and 9 ($P < 0.001$), and insulin ($P < 0.05$) and glucagon ($P < 0.025$) release increased slightly.

B-Cell function was examined further in D-valine medium and following BUdR treatment. Glucose-stimulated insulin release was maintained on culture Day 6 following two

TABLE II. INSULIN SECRETION BY BUdR-TREATED CULTURES[a]

Hour[b]	Glucose (mM)	n	Insulin (μU/ml ± SE)	P[c]
1	1.7	6	413 ± 29	
2	1.7	6	487 ± 44	
3	1.7	6	448 ± 11	
4	1.7	6	415 ± 24	
5	16.5	6	764 ± 52	<0.001

[a] Cultures were incubated in Eagle's MEM containing glucose (5.5 mM), gentamicin (50 μg/ml), 10% (v/v) heat-inactivated fetal bovine serum, and BUdR (10^{-5} M) for 24 hr on culture Day 2 and again on culture Day 4. Following each incubation with BUdR, cultures were exposed to fluorescent light for 90 min. Cultures were incubated in 45% (v/v) NCTC-135, 45% (v/v) Med 199, and 10% (v/v) heat-inactivated fetal bovine serum containing glucose (16.5 mM) and gentamicin (50 μg/ml), between incubations in BUdR medium.

[b] On culture Day 6, cultures were incubated for successive 1-hr periods in 45% (v/v) NCTC-135, 45% (v/v) Med 199, and 10% (v/v) heat-inactivated fetal bovine serum containing glucose at the indicated concentrations, and gentamicin (50 μg/ml), and insulin released into the medium during each 1-hr period was measured. Medium was changed hourly.

[c] P vs all other groups.

TABLE III. INSULIN SECRETION IN D-Val MEDIUM[a]

Glucose (m*M*)	Experiment 1			Experiment 2		
	n	Insulin (μU/ml ± SE)	*P*[c]	*n*	Insulin (μU/ml ± SE)	*P*[c]
1.7	2	733 ± 13		10	127 ± 4	
1.7	2	613 ± 13		10	150 ± 4	
16.5	2	1378 ± 12	<0.001	10	333 ± 15	<0.001
1.7	2	568 ± 47		10	133 ± 10	

res were incubated in Eagle's MEM containing glucose (16.5 m*M*), gentamicin (50 μg/ml), nonessential ds, 10% (v/v) heat-inactivated and dialyzed fetal bovine serum, and D-Val (92 mg/liter) substituted for L-

lture Day 8, cultures were incubated for successive 1-hr periods in Eagle's MEM containing glucose at the concentrations and other additions as noted above, and insulin released into the medium during each 1-hr s measured. Medium was changed hourly.
ll other groups.

atments with BUdR (Table II). Like- ıcose-stimulated insulin release was ɔn culture Day 8 in cultures that had intained in D-valine medium (Table

ɪsion. The usefulness of pancreatic /er cell cultures in the long-term ' islet cell function has been limited ıe rapid proliferation of fibroblastoid ıe rapid growth of fibroblasts is un- ɛ not only due to their mechanical g effect upon the endocrine cells but ause of their consumption of nutri- though a number of methods have ɔorted to be useful in decreasing the of fibroblasts in these cultures, the ısms by which these effects were pro- ɛmain unexplained.

ı been clearly demonstrated in the reported here that cell proliferation ıced much more than either insulin gon release under the culture condi- ted. It has been previously reported y cells containing D-amino acid oxi- ı convert D-valine to the essential L- ner (7). We have not been able to mically confirm the presence of D- cid oxidase in these cultured islet cells ished). Nevertheless, it is apparent ɛreas culture DNA content is greatly by substitution of D-valine for L- oth glucagon and insulin release are ıced as much. This suggests that A- ɛll function have not been affected as ı culture growth by maintenance of tic cells in this medium. These find- vell as the previously reported effects ically defined medium (2), cystine-deficient medium (3), and addition of sodium ethylmercurithiosalicylate (4, 5) suggest that a common feature underlying these purification approaches may be the apparently less fastidious requirement for certain medium constituents by the endocrine cells than the more rapidly proliferating fibroblasts. This may also explain why these inhibitory conditions have been observed to be less effective when the fibroblasts become confluent and contact inhibited, a situtation in which fibroblast proliferation is reduced.

The successful application of BUdR is also based upon the property of the fibroblasts to proliferate more rapidly than do the endocrine cells. Following incubation of subconfluent pancreatic monolayer cultures for 24 hr with [^{3}H]thymidine, greater than 95% of the fibroblasts exhibit labeling of nuclei compared to a 5–7% labeling index for endocrine cells (unpublished (21, 22)). Like thymidine, BUdR is incorporated into DNA by proliferating cells, and this proves lethal when these cells are subsequently exposed to fluorescent light (8). Thus when subconfluent pancreatic cultures are treated for 24 hr with BUdR, preferential incorporation of BUdR by fibroblasts may be expected to lead to a loss of greater than 95% of the fibroblasts, compared to a loss of about 5% of the islet cells. Furthermore, when BUdR-treated cultures are subsequently maintained in control medium, recovery of proliferation may be expected, as indicated by a rise in DNA content, while islet cell numbers are expected to remain quite constant, as indicated by amounts of insulin and glucagon released. Indeed this appeared to be the case when the

data in Table I was examined. Although not tested specifically, these results suggest that sequential treatments with BUdR may be more useful than a single treatment for the continued reduction of fibroblast proliferation in these pancreas cultures.

Summary. The use of either D-valine-substituted medium or BUdR treatment have been shown to be effective in reducing fibroblastoid cell proliferation in pancreatic monolayer cell cultures established from neonatal rats. Maintenance of these cultures in D-valine medium appeared to completely curtail further culture growth, but this was apparently accompanied by a loss of both A and B cells as evidenced by a fall in both glucagon and insulin release. A single 24-hr treatment of cultures with BUdR was just as effective in curtailing fibroblastoid cell growth, but within a few days following such treatment, fibroblast proliferation resumed, although the number of fibroblasts remained reduced compared to control cultures. On the other hand, with BUdR treatment continued loss of A and B cells apparently did not occur.

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degree Celsius (Centigrade)	°C	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	diam	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
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inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
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ɔs OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 245–248 (1979)

:tion of Hypertension in One-Kidney One-Clip Rabbits by Immunization with Tonin (40658)

'L GARCIA, ERNESTO L. SCHIFFRIN, JOLANTA GUTKOWSKA, ROGER BOUCHER AND JACQUES GENEST

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in, an enzyme of the serine–protease , is present in various rat tissues, espe- submaxillary glands and kidneys. It s angiotensin II directly from angi- ıogen, from the synthetic tetradecapep- nin substrate and from angiotensin I This new enzyme has been purified s physicochemical characteristics and ıte specificity are well established (3, ıas been crystallized by Hayakawa *et*

fall in blood pressure after adiminis- of angiotensin II antibodies or angio- antagonists (saralasin) seems to indi- ıat the renin–angiotensin system is in- in the pathogenesis of the two-kidney, p Goldblatt hypertension (one renal clamped, contralateral kidney un- d) (6–8).

direct role of the renin–angiotensin in inducing or maintaining elevated pressure in the experimental one-kid- ne-clip Goldblatt hypertension (one ırtery clamped, contralateral nephrec- remains a highly controversial subject. immunization against angiotensin II own to be ineffective in preventing the pment of this type of hypertension in ; (9, 10). Neither the administration of ıin serum (11), angiotensin II synthetic nist (12), nor converting enzyme inhib- .3) have been effective in lowering the pressure in this model of experimental ension.

lies from our laboratory (14) have dem- ed that the blood pressure of rats with dney one-clip hypertension can be de- l to normal levels by passive immuni- with rabbit tonin antibodies. These findings, together with the availability : tonin, led us to investigate the effect ve immunization with this enzyme in :-kidney one-clip hypertensive rabbit.

Materials and methods. Male New Zealand white rabbits (2 to 2.5 kg) were kept in a constant-temperature room, fed with Purina rabbit chow, and allowed free access to tap water.

After a control period of I week, the right kidney was removed under anesthesia (sodium pentobarbital, Nembutal, 30 mg/kg i.v.) through a flank incision.

One week later the left renal artery was constricted with a silver clip (15) having an internal gap of 0.58 mm. Blood pressure was measured at room temperature (22°) twice a week, indirectly in the central ear artery using the capsule of Grant and Rothschild (16). The average of three readings was recorded.

The animals were considered hypertensive when blood pressure was consistently at least 25 mm Hg higher than control values.

Eleven rabbits with hypertension of 3–5 weeks' duration were injected subcutaneously at several sites with a single dose of 50 μg of purified rat tonin (3). Tonin was diluted in 0.5 ml of 0.9% NaCl and well mixed with an equal volume of Freund's complete adjuvant. Blood was withdrawn every 2 weeks.

Serum antitonin was measured by radioimmunoassay (17). The incubation mixture consisted of 100 μl of ^{125}I-labeled tonin containing about 6000 cpm and 100 μl of antiserum at 1:1000 dilution.

The tubes were incubated at 4° for 24 hr. The antibody-bound tonin was precipitated by adding 50 μl of goat anti-rabbit γ-globulin diluted 1:50 in normal rabbit serum 1:1000 and incubating for 48 hr at 4°. The precipitates were isolated by centrifugation at 4000 rpm for 20 min at 4° and their radioactivity was determined in a gamma counter.

Serum antitonin was expressed as the percentage of bound labeled tonin.

Plasma renin activity was measured by radioimmunoassay of generated angiotensin I

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(18). The data are expressed as mean values ± SD. Comparisons were made by the paired or unpaired *t* test.

Results. The effect of immunization with 50 μg of purified tonin on rabbit No. 52 is shown in Fig. 1. After right nephrectomy and left renal artery constriction blood pressure rose till sustained levels were reached. Four weeks after the rabbit became hypertensive, tonin was injected. One week later the blood pressure has fallen and it attained preclamping values 2 weeks after the injection. Antibodies against tonin were detectable 20 days after immunization; they reached their highest titer 80 days later and gradually decreased thereafter. We could not use more concentrated plasma to increase the sensitivity of our assay for antitonin antibodies because of the presence in plasma of an interfering nonimmune protein which binds tonin. This may account for the discrepancy in time between the reduction in blood pressure and the detection of antibodies. Blood pressure remained below preimmunization levels throughout the observation period, increasing slightly 60 days after immunization. There was no apparent correlation between antibody titer and decrease in blood pressure.

Figure 2 relates to rabbit No. 84, in which the tonin injection did not elicit antibody formation. Blood pressure continued to rise after tonin injection in this case. Two groups of animals falling into the two different patterns, were clearly defined (Table I). Out of 11 hypertensive rabbits with a blood pressure of 83 ± 7 mm Hg injected with tonin, 5

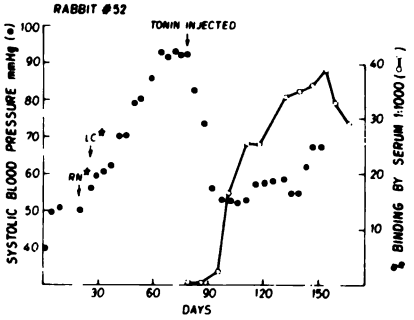

FIG. 1. Effect of the injection of tonin on blood pressure in a one-kidney, one-clip hypertensive rabbit (No. 52). RN = right nephrectomy, LC = left renal artery clipped. (●) BP, average of three readings; (○) antibody titer.

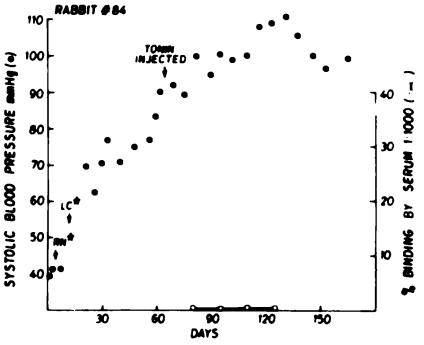

FIG. 2. Effect of the injection of tonin on blood pressure in a one-kidney, one-clip hypertensive rabbit (No. 84). Abbreviations and symbols as in Fig. 1.

responded with antibody formation (responders) and in all of these the blood pressure had decreased significantly at the end of 4 weeks from 86 ± 5 mm Hg to 60 ± 9 mm Hg ($P < 0.001$). In the second group, in which tonin injection was not followed by antibody formation (nonresponders), the blood pressure further increased over the corresponding period from 82 ± 9 mm Hg to 95 ± 8 mm Hg ($P < 0.001$). No significant differences were found between responders and nonresponders for the comparison between control and preimmunization blood pressures, but the difference was highly significant ($P < 0.001$) for the comparison 4 weeks and 3 months after immunization.

At the end of an observation period of 5 months the blood pressure in the responder group was further, although not significantly, reduced to 55 ± 10 mm Hg (not significantly different from controls), while in the nonresponder group it increased to 116 ± 13 mm Hg ($P < 0.001$).

No difference was found in plasma renin activity (PRA) 4 weeks after tonin injection between both the two groups: 2.13 ± 0.9 ng AI/ml·hr^{-1} in responders compared to 1.92 ± 1.3 ng AI/ml·hr^{-1} in nonresponders.

Discussion. These observations show that one-kidney one-clip hypertensive rabbits are clearly separated into two groups after being injected with tonin. The fall in blood pressure produced in five animals coincided with the appearance of antitonin antibodies; in none of the animals in which antibodies were not detected was the blood pressure lowered. The normalization of the blood pressure in the responder group lasted throughout the period

TABLE I. EFFECT OF ACTIVE IMMUNIZATION WITH TONIN ON ONE-KIDNEY, ONE-CLIP HYPERTENSIVE RABBITS

	Before immunization		After immunization			
Rabbit	Control BP (mm Hg)	BP before injection (mm Hg)	At 4 weeks BP (mm Hg)	Ab titer %	At 3 months BP (mm Hg)	Ab titer %
51	36	79	53	8	41	57
52	48	93	55	17	55	29
55	56	82	59	5	67	20
56	60	86	60	9	61	40
63	53	88	75	24	53	51
Mean ± SD	51 ± 9	86 ± 5	60 ± 9		55 ± 10	
84	39	81	91	0	111	—
86	34	72	81	0	111	—
87	49	98	104	0	129	—
91	47	80	95	0	99	—
94	44	80	99	0	130	—
6	41	78	102	0	—	—
Mean ± SD	42 ± 6	82 ± 9	95 ± 8		116 ± 13	

during which antibodies were being produced. This strongly suggests that tonin may be responsible for the development and maintenance of hypertension in one-kidney one-clip rabbits.

These observations are consistent with the fall in blood pressure observed in one-kidney one-clip hypertensive rats after they had been injected with antitonin antibody (14) and with the results of Kondo *et al.* (19), who described both potentiation to norepinephrine and a direct vasoconstriction when tonin was infused into the rat mesenteric preparation. These effects were not blocked by a synthetic angiotensin II antagonist, which suggests that tonin acts either upon a protein substrate in the endothelial cell membrane to generate angiotensin II *in situ*, or directly on the arterial wall. These findings could explain the lack of effect of angiotensin II antibodies or of angiotensin II antagonists in the one-kidney one-clip hypertensive animals (9–13).

It is interesting to note that a significant decrease in blood pressure in renovascular hypertensive animals has been reported after the injection of crude kidney homogenates (20–23).

Summary. Male New Zealand white rabbits with one-kidney one-clip Goldblatt hypertension of 3–5 weeks' duration were injected with purified tonin. Of 11 injected animals, 5 developed antibodies and in all of these animals blood pressure returned to normal levels. No change in blood pressure was observed in 6 animals in which no antibodies were detected. These findings are consistent with previous studies in rats and suggest that tonin plays a significant role in maintaining high blood pressure in one-kidney hypertensive animals.

The authors wish to thank Mrs. Suzanne Diebold, Miss Louise Lamarre, Miss Sylvie Duniol, and Mr. Michel Chamberland for the technical assistance and Mrs. Vivianne Lacombe for secretarial work. This work was supported by a Medical Research Council of Canada Group Grant to the Hypertension Multidisciplinary Group of the Clinical Research Institute of Montreal and the Quebec Heart Foundation (Dr. J. Gutkowska).

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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 249–253 (1979)

Evidence of Breakdown of Pneumococcal Polysaccharides *in Vivo* (40659)[1]

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'saccharides are being increasingly em-
l as vaccines for the prevention of se-
›acterial infections because of their low
y and good immunogenicity in man.
ıococcal polysaccharides are among
:ent additions to this armamentarium.
is still much to be learned about the
ccharides as immunogens, however, in-
g the duration of the immunity they
and the eventual metabolic fate of the
ns. Pneumococcal capsular polysaccha-
(PCP) are known to be resistant to
lation *in vivo*. This resistance is evident
heir prolonged persistence in reticulo-
ıelial tissues in studies of immunologi-
ɛrance (1). The persistence of polysac-
e in animals injected with large doses
P in tolerance studies, however, does
eclude the possibility that these anti-
re slowly degraded *in vivo*. In an anal-
situation, Janeway and Humphrey (2)
›bserved that "indigestible" D-amino
olypeptides are metabolized in mice,
! times more slowly than the corre-
ng L-amino acid polypeptides. In the
t studies, high-molecular-weight type
pe 8 PCP were injected into rats, and
ce of their breakdown was obtained by
ying low-molecular-weight derivatives
antigens in the urine.

erials and methods. Adult, male,
ıe–Dawley rats weighing an average of
were used. Rats were fed 5% sucrose
for 2 h, after which they were placed in
›lic cages for 6 hr for collection of
Serum was obtained by bleeding from
l vein.

e 1 and type 8 PCP for injection (Lots
! and 812643F, respectively) were ob-
from Eli–Lilly & Company, Indian-
Indiana. The antigens contained less
5% contaminating protein, nucleic acid
·substance.

Chromatography of PCP was performed with a 0.5-ml sample size on Sephadex G-200 with a 0.9 × 60-cm column equilibrated with 0.1 *M* Tris (Trishydroxymethylaminomethane)-HCL buffer (pH 8.0) and 0.02% Na azide. The void volume was measured with blue dextran (2×10^6 mean M_r), and columns were calibrated with dextrans T20 (M_r 20,000), T40 (M_r 40,000), and T70 (M_r 70,000) (Pharmacia, Inc., Piscataway, N.J.). Eluate fractions of 0.5 ml were collected at a flow rate of 4.5 ml/hr, and dextran in the eluate was determined by the method of Mokrasch (3). PCP in eluate fractions was detected by counterimmunoelectrophoresis (CIE), as described elsewhere (4). The sensitivity of this assay is 0.1 μg/ml. Type-specific rabbit antipneumococcal sera (Statens Seruminstitut, Copenhagen, Denmark) was used throughout. Precipitin lines were developed for 24–48 hr at 5°.

For gel-precipitin analysis of PCP, solutions containing antigen were dialyzed against distilled water at 5° in cellulose bags (retention size > 12,000 M_r, Arthur H. Thomas Co., Philadelphia, Pa.). Samples were concentrated 5- to 40-fold by lyophilization and reconstituted in normal saline. Double diffusion was carried out with plates coated with 1% agarose in barbital–barbital-sodium buffer (pH 8.2). Precipitin reactions were developed at room temperature and read at 72 hr. Immunoelectrophoresis was performed for 1.5 hr under the same electrical conditions as were used for CIE. Antiserum was added to troughs cut equidistant between the wells, and precipitin reactions were developed for 24–48 hr.

PCP, IgG, and IgM in rat kidney were evaluated by indirect immunofluorescence, as described in detail elsewhere (5). Briefly, tissue was quick frozen and sections of 4–5 μm were cut on a cryostat. Optimal dilutions of rabbit or goat antisera (Cappel Laboratories, Cochranville, Pa.) were applied for 15

ported by the Veterans Administration.

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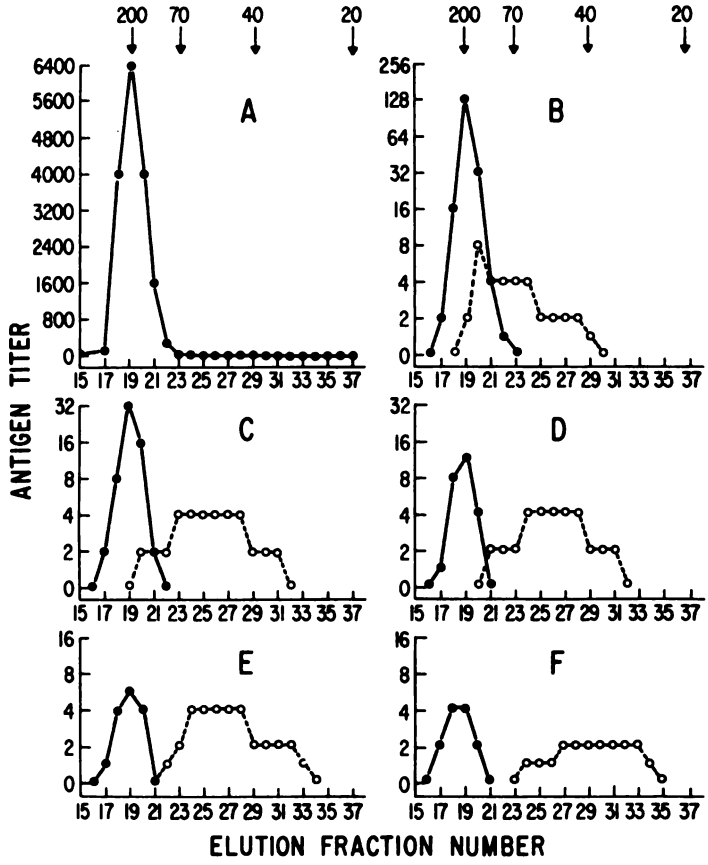

FIG. 1. Chromatograph of type 8 PCP on G-200 before injection (A) into rats and in serum (●) or urine (○) at 3 (B), 6 (C), 14 (D), 21 (E), and 35 (F) days after injection.

min at room temperature, followed by three washings of 15 min each with phosphate-buffered saline. Controls included the use of antipneumococcal sera of a heterologous type, normal goat, or rabbit sera, and the use of fluoresceinated antisera alone. Normal tissue controls were also employed.

Results. Type 8 PCP vaccine for use in rats was chromatographed on Sephadex G-200 in order to free it of low-molecular-weight polysaccharide. In this procedure, 1.0 mg of PCP was dissolved in 0.25 ml of normal saline and applied to a precalibrated column. Eluate fractions of 0.5 ml were collected and the antigen titer was determined by CIE. The bulk of the antigen eluted in the void volume of the column. Fractions representing high-molecular-weight PCP (≥70,000), as indicated by column markers, were pooled, lyophilized, and redissolved in 0.25 ml of sterile water. The pooled fractions were chromatographed a second time on Sephadex G-200. A typical pattern from this elution is indicated in Fig. 1A. The appropriate fractions were again pooled and lyophilized. The pooled antigen was dialyzed against phosphate-buffered saline (pH 7.2) and brought to a volume of 1.0 ml for injection. Titration by CIE of preparations of doubly chromatographed PCP indicated that ~80% of the initial PCP was recovered as high-molecular-weight antigen for injection.

Each of four rats were injected i.p. with ~800 μg of doubly fractionated type 8 PCP. Serum and urine were collected serially and chromatographed on Sephadex G-200 to measure molecular weight. Results were essentially identical for all animals. Data for a representative animal are depicted in Fig. 1. The mean molecular weight of the doubly fractionated PCP before injection was ≥200,000, with no detectable antigen of

000 (Fig. 1A). Chromatographs of PCP ine collected at 72 hr after injection (Fig. revealed small amounts of PCP in the cular weight range of 40,000–70,000. chromatograph of PCP in urine at 72 hr lapped appreciably with the chromato- h of the PCP in the serum sample taken hr. There was progressively less overlap een chromatographs of serum and urine in samples taken at subsequent intervals 1C–1F). Serial samples of urine ob- d over a 35-day period consistently con- d PCP of <70,000 M_r. In contrast, PCP ıe serum throughout this period was),000 M_r. Immunologic analysis of type P in the urine showed that it differed doubly fractionated type 8 PCP vaccine. ɔuble immunodiffusion in agarose, urine gave a reaction of partial identity with /accine (Fig. 2). In immunoelectropho- (Fig. 3), urine PCP displayed more rapid al migration than did vaccine PCP. PCP rum obtained at 6, 14, and 21 days after tion was immunologically identical with ıne PCP in double-immunodiffusion es.

ldditional studies were carried out using 1 PCP rather than type 8 PCP. The ;en was doubly chromatographed on adex G-200, after which it had a mean f ≥200,000 and contained no antigen of 000 M_r. Each of six rats were injected with ~1200 μg. Results of chromato- hs of the serum and urine of these ani- are indicated in Table I. After injection, appeared very promptly in the urine (as as 3 hr in one spot check) where it sted during 21 days of observation. Early

i. 2. Comparison of PCP in rat urine (A) and e PCP (B) by use of double immunodiffusion. PCP was obtained from a rat injected 12 days with type 8 vaccine. The upper well was filled /pe 8 rabbit antipneumococcal antibody.

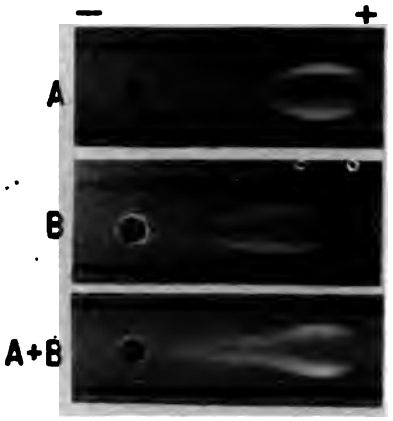

FIG. 3. Immunoelectrophoresis of rat urine PCP (A), vaccine PCP (B), or a mixture of urine and vaccine PCP (A+B). Urine PCP was obtained from a rat injected 12 days earlier with type 8 vaccine. Troughs were filled with type 8 antibody.

TABLE I. MOLECULAR SIZE OF PCP IN THE SERUM AND URINE OF SIX RATS INJECTED WITH ~1200 μg TYPE 1 PCP

Days	No. rats studied	Body fluid	Range of molecular size of PCP: ≥200,000 to 70,000	70,000 to 40,000	40,000 to 20,000
3	2	Serum	X		
		Urine	X	X	X
7	1	Serum	X		
		Urine	X	X	X
13	2	Serum	X		
		Urine		X	X
21	1	Serum	X		
		Urine		X	X

samples of urine contained PCP >70,000 M_r, but this relatively high-molecular-weight antigen disappeared from the urine by 13 days. As was observed with type 8 antigen, the urine of the injected animals consistently contained antigen of 20,000–70,000 M_r. Type 1 PCP in the serum was of high-mean-molecular weight (≥200,000) with no PCP of <70,000.

In a third study, rats were injected with 1500 μg of type 1 PCP, and renal tissue was evaluated with indirect immunofluorescence to determine the levels of PCP in the tissue over a 49-day period. Results are listed in Table II. Aggregates of polysaccharide were detected principally in the glomerular mesangium and to a lesser extent in renal capil-

TABLE II. RESULTS OF TESTS FOR ANTIGEN IN 20 RATS INJECTED WITH 1500 μg OF TYPE 1 PCP

Days	No. rats	No. with positive renal fluorescence			No. with PCP in urine	Serum PCP (μg/ml)
		PCP	IgM	IgG		
0	2	0	0	0	0	<0.1
1	2	2	0	0	2	128
2	2	2	0	0	2	80
3	2	2	1	0	2	68
6	2	2	1	0	2	24
10	3	3	1	0	3	—
14	2	2	0	0	2	4
28	3	1	0	0	3	2
49	2	0	0	0	0	1

lary endothelium. The amount of PCP in the kidneys increased during the first several days after injection and then declined. By 28 days, only one of three rats had detectable type 1 PCP in the kidneys, and two rats examined at 49 days had no PCP. The decrease in detectable PCP was not due to masking by specific antibody produced by the animals, because neither IgM nor IgG could be detected in sections of kidney obtained after 14 days.

Discussion. The present studies indicate that when high-molecular-weight PCP is injected i.p. into rats, antigen is excreted promptly in the urine. Some of this excreted antigen is of high molecular weight (i.e., >70,000), and because of its very rapid appearance in the urine probably represents PCP which passes unaltered through the kidneys. It has been reported that type 3 PCP with a mean M_r of 150,000 is excreted readily in the urine of mice after i.p. injection (6). Type 8 PCP (and probably type 1 PCP) resembles type 3 PCP in that it is a simple, unbranched molecular structure; this fact may explain why large molecules of the antigen are readily excreted by the kidney (6, 7).

Low-molecular-weight PCP (<70,000) is also present in the urine soon after injection of vaccine PCP, and this low-molecular-weight antigen remains detectable long after the larger-molecular-weight PCP has disappeared from the urine. It is possible, although unlikely, that this antigen originated as a trace contaminant in the injected PCP. If this were true one might expect that all of the low-molecular-weight PCP would have been rapidly excreted, but low-molecular-weight PCP was excreted for weeks after the higher-molecular-weight PCP (which certainly constituted the bulk of the injected material) had disappeared from the urine. Also, type 8 PCP in the urine showed only partial identity with vaccine PCP, and urine PCP showed significant differences from vaccine PCP in immunoelectrophoresis. Taken together, these observations strongly suggest that low-molecular-weight PCP in the urine is a degradation product of the injected antigen.

The conclusions from the present study are consistent with observations by Jones *et al.* (6) who found that mice injected with ^{125}I-labeled tyramine–type 3-polysaccharide excreted small amounts of ^{125}I-labeled tyramine–glucose in the urine, and the ^{125}I-labeled polysaccharide that was excreted in the urine did not bind type 3 antibody as well as the original antigen. These findings were interpreted by the authors as evidence of degradation.

Several investigators have documented that injected PCP gradually disappears from animal tissues. Stark (8, 9), for example, noted that there was a marked decrease with time in quantity of antigenic ^{14}C-type 1 PCP in the spleens of mice. Kaplan and Coons, who used immunofluorescence techniques (10), showed a gradual decrease in detectable PCP in tissues of mice injected with types 2 or 3 antigens. The present studies with immunofluorescence confirm their observations and indicate that the decline in detectable PCP is not due to masking by endogenously produced antibody. The findings offer further support for the concept that there is gradual degradation of PCP *in vivo*.

If the present observations are applicable to man, one would expect that pneumococcal polysaccharides would be subject to breakdown in vaccinated subjects. Because the immune response to PCP may depend on stim-

ı of B lymphocytes by persisting poly-
ride (1), degradation of the antigens
ɛ associated with waning of immunity.
ability of polysaccharide antigens *in*
ppears to be an additional factor to
er in assessing polysaccharide vaccines.
mary. Type-specific PCP vaccines
1 and 8) were fractionated twice on
lex G-200 to eliminate antigen of
0 M_r and the vaccines were injected
ɔ rats. PCP of <70,000 M_r was excreted
urine within 72 hr after injection, and
1 week most of the PCP in the urine
70,000 M_r. PCP in the urine had in-
l electrophoretic mobility and showed
ırtial immunologic identity in compar-
ith the injected PCP. Deposits of PCP
enal mesangium and endothelium de-
with time and were eventually elimi-
from the tissue. These data indicate that types 1 and 8 PCP are slowly degraded in rats.

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Paradoxical Induction of Tyrosine Aminotransferase by Progesterone *in Vivo*: Potentiation of Effects of Low Levels of Glucocorticoid Hormone (40660)

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The ability of a variety of natural and synthetic steroids to antagonize glucocorticoid hormone action at the receptor level is now well established (1, 2). A number of such antiglucocorticoid compounds have been studied in detail by Tomkins and his coworkers, who postulated that they have affinity for a different conformational form of the cytoplasmic glucocorticoid receptor from that to which true glucocorticoids bind (2), such that the complex formed between receptor and antiglucocorticoid is incapable of activation and subsequent transfer to the nucleus (3).

A diversity of antiglucocorticoid hormonal effects have now been examined in *in vitro* studies (1–8), the majority of which have employed progesterone. It has been shown that, in keeping with its ability to bind to the cytosolic glucocorticoid receptor (2) and the inability of the resulting steroid–receptor complex to be translocated to the nucleus (3), progesterone is an effective antagonist to a number of well characterized responses to glucocorticoids *in vitro*. Thus progesterone or other antiglucocorticoids have been shown to block glucocorticoid-induced stimulation of tyrosine aminotransferase (TAT) synthesis by hepatoma cells in tissue culture (1), to antagonize glucocorticoid-mediated cytolysis (7) or inhibition of glucose uptake (5) in thymus cells, to reverse the induction of alkaline phosphatase in HeLa cells by prednisolone (6), and, most recently, to overcome the glucocorticoid hormone-induced inhibition of hepatoma cell proliferation *in vitro* (8).

Despite considerable interest in the subject, virtually no studies thus far have examined possible antiglucocorticoid effects of progesterone *in vivo*. Although, because of its other biological effects, there might well prove to be problems associated with the use of progesterone as a therapeutic antiglucocorticoid, the possibility that at the very least it might prove of use as an experimental pharmacologic tool made it of interest to explore its potential properties as an antiglucocorticoid in intact animals. In this paper we report the results of studies initially undertaken to examine the effects of progesterone as a potential *in vivo* antagonist to the well-defined phenomenon of glucocorticoid-induced tyrosine aminotransferase synthesis in rat liver. In contrast to the anticipated antagonistic effect, we were surprised to find that progesterone exerts a striking synergism upon the induction of this enzyme by exogenous glucocorticoid, and in addition markedly stimulates the synthesis of TAT in response to endogenous circulating glucocorticoid. These observations, as well as an investigation of possible mechanisms by which this potentiation is mediated, form the basis for this report.

Materials and methods. Materials. Studies were performed on male Sprague–Dawley rats weighing between 130 and 150 g. Normal animals were maintained on food and water *ad libitum*, and adrenalectomized rats on the same regimen except for supplementation of their drinking water with 0.9% NaCl. Adrenalectomies were performed by the Zivic-Miller Laboratories, Inc. (Allison Park, Pa.), and subsequent studies were performed between 5 and 10 days after adrenalectomy. Hydrocortisone, progesterone, and dexamethasone were all obtained from the Sigma Chemical Company (St. Louis, Mo.).

Administration of steroids and preparation of samples for TAT assay. All steroids (in doses expressed per 100 g of body weight) were administered in solution by intraperitoneal injection in 0.3 ml dimethylsulfoxide (DMSO). Control animals received an equal volume of DMSO alone. All groups contained four to six animals each. Rats were injected between 9 and 10 AM and were sacrificed by stunning and exsanguination exactly 4 hr later. Typical dose–response relationships for the induction of TAT by hydrocortisone and dexamethasone are shown in

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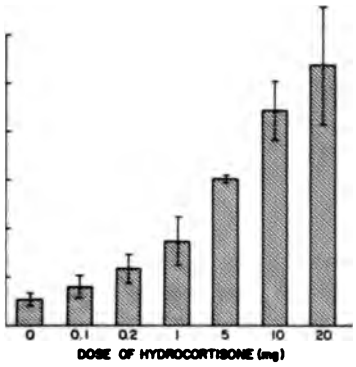
nd 2, respectively. DMSO by itself atedly shown to have no effect on Γ activity.

ing sacrifice, livers were immediioved and homogenized individually of chilled 0.14 *M* KCl, and all subteps were performed at 0–4° unless noted. Homogenization was aced by means of 20 strokes in a ype, hand-driven, all-glass homoguipped with a loosely fitting pestle g Glass Specialties, Rochester, Iomogenates were centrifuged at for 30 min, and the resulting clear unt fractions decanted into individd tubes prior to assay for TAT ac-

ie aminotransferase activity. This was ed by the method of Diamondstone minor modification. One unit of aminotransferase activity was dehat quantity of enzyme resulting in ation of 1 nmole of product per 37° under saturating substrate con- "working extinction coefficient" of l^{-1} for *p*-hydroxybenzaldehyde at as used (9).

. *Initial studies in normal animals: n" of TAT by progesterone alone.* examination of progesterone as a antagonist to the induction of TAT nous glucocorticoid, the effects of oses of progesterone alone were ex-



Induction of tyrosine aminotransferase function of hydrocortisone dose. Hydrocore doses indicated on the abscissa was adminadrenalectomized rats and hepatic TAT activned 4 hr later as described under Methods. induction is achieved at a dose of 10 mg one.

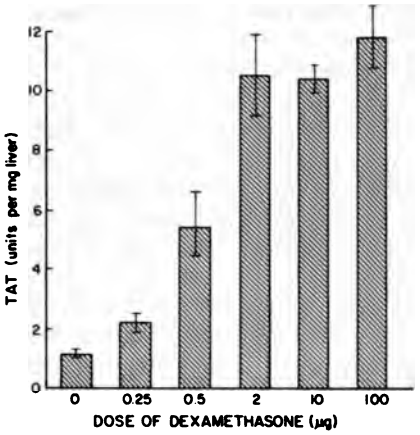


FIG. 2. Relationship between dexamethasone dose and TAT induction in adrenalectomized rats. Maximal induction is observed at a dose of 2 μg dexamethasone.

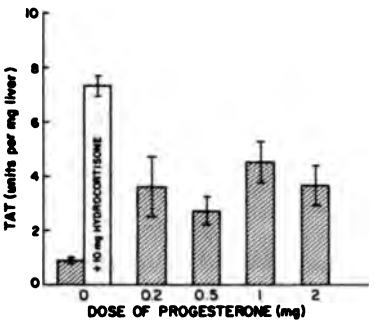


FIG. 3. Induction of TAT in normal rats by the administration of progesterone. The dose of progesterone is given on the abscissa, and, for comparison, the effect of a maximally inducing dose of hydrocortisone (10 mg) is included. Note that about half-maximal induction is achieved by the administration of as little as 0.2 mg progesterone, and that increasing this dose by a factor of 10 is without further effect.

amined in normal animals (adrenal glands intact). A surprising finding was that, instead of being without effect, or possibly even causing a fall in liver enzyme activity due to a peripheral antagonism to endogenous glucocorticoid (corticosterone), the administration of progesterone by itself resulted in a striking rise in hepatic TAT activity (Fig. 3). The effect was fully evident at doses of progesterone as low as 0.2 mg, showing no increase even with doses an order of magnitude higher.

Studies in adrenalectomized animals: Potentiation of low doses of exogenous glucocorticoid by progesterone. Because of the unex-

pected finding that progesterone was a potent inducer of TAT in normal rats, the possibility that progesterone might itself possess intrinsic glucocorticoid activity *in vivo* was tested and excluded by experiments in adrenalectomized animals. Figure 4 shows that, in contrast to the effects of progesterone administration in intact animals (cf. Fig. 3), even large doses of progesterone, when administered to adrenalectomized rats, were found to be entirely without effect upon liver TAT activity. What was of special interest, however, was that, while inactive in these latter animals when given by itself, progesterone—even at low doses—markedly potentiated the induction of enzyme by submaximal doses of simultaneously administered glucocorticoid. In the presence of progesterone, a dose of 0.2 mg hydrocortisone—itself barely sufficient to cause a significant elevation in enzyme activity—is seen to result in a level of induction achieved only with a maximally inducing (50-fold higher) dose of hydrocortisone alone (see also Fig. 1).

Disappearance of progesterone effect at large doses of exogenous glucocorticoid. The observation that the induction of TAT by progesterone alone is completely blocked by antecedent adrenalectomy, and the further finding that inducibility can be restored by

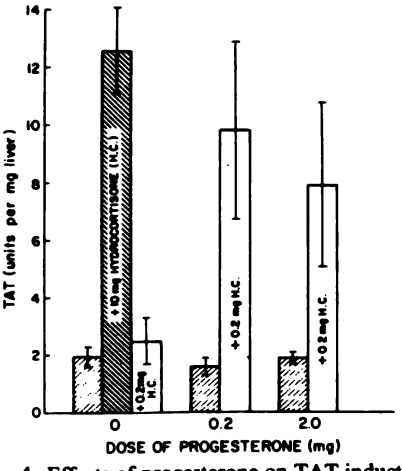


FIG. 4. Effects of progesterone on TAT induction in *adrenalectomized* animals. While progesterone by itself is seen to be entirely without effect upon TAT activity in adrenalectomized rats, it strikingly potentiates the induction of enzyme by low doses of hydrocortisone (H.C.; see text). The effect of a maximally inducing dose of hydrocortisone is again included for comparison.

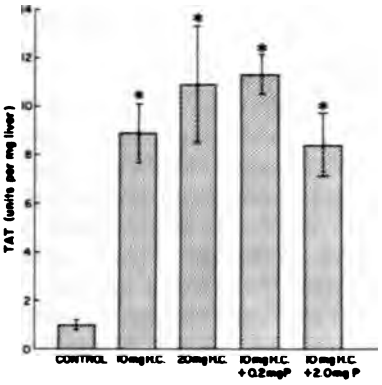
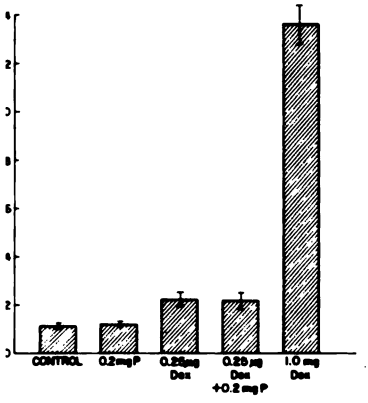


FIG. 5. Absence of a potentiating effect of pr one (P) at high (maximally inducing) doses of hy tisone (H.C.) in adrenalectomized rats. None of th values for bars marked with asterisks differ signi from each other ($P > 0.1$).

simultaneous repletion with a small d glucocorticoid that by itself has only a mal effect on enzyme induction, sugge number of possible interpretations. On possibility was that the rise in TAT induced by progesterone, rather than i ing a stimulation of enzyme synthesis, instead be attributable to a reduction rate of which TAT, once synthesized, graded. Although unlikely *a priori* (t crease in TAT activity following proges administration is large in magnitude a curs rapidly with respect to the norma life of the enzyme in liver (10)), this pc ity was nevertheless tested by examini influence of progesterone administrat the levels of TAT induced by large d hydrocortisone. If, rather than stimu TAT synthesis, the administration of p terone interfered with the degradat TAT by some independent mechanis potentiating effect of the hormone ob at low doses of glucocorticoid should served at large doses of glucocorticoid a That this is not the case is seen in which shows that at large doses of hyc tisone the simultaneous administrat progesterone is entirely without add effect upon hepatic TAT activity.

Possible relation of observed synerg competitive binding of progesterone an ral glucocorticoids by transcortin: Cont absence of synergism between progesterc dexamethasone. An additional mecl



Lack of synergism between progesterone (P) nethasone (Dex). The absence of synergism ·ogesterone and dexamethasone contrasts with ation by progesterone of enzyme induction in ɔ endogenous corticosterone (Fig. 3) and ex- ydrocortisone (Fig. 4).

ı contrast to the preceding one, might for a true potentiation of glucocorti- uced TAT synthesis by progesterone, :h would be consistent with all of the g experimental observations, would ıation of plasma glucocorticoid bind- by progesterone: Progesterone, a hor- th known high affinity for transcor- ht exert its effect by binding to the rotein and thereby either displacing ly bound endogenous corticosterone nting the binding of exogenous hy- ıone with a resulting marked rise in ɛntration of free (unbound) gluco- in the plasma, and a corresponding tracellular glucocorticoid concentra- s possibility is directly testable, for it a specific experimental prediction. If ıe mechanism by which progesterone tes the effect of exogenous hydrocor- · endogenous corticosterone is attrib- o a competition for glucocorticoid sites on transcortin and a consequent ɛe glucocorticoid concentration, one ɛdict that the phenomenon of poten- hould be *absent* in the instance of a ticoid such as dexamethasone which, ıst to the former two glucocorticoids, t bind to transcortin (2, 15). The

ffinities of progesterone, corticosterone, and ıone for transcortin are 90, 30, and 30 μM^{-1}, r (11).

effects of progesterone given simultaneously with a dose of dexamethasone by itself sufficient to result in a definite but only modest increase (a doubling) of enzyme activity were accordingly examined. Figure 6 shows the result. In marked contrast to its synergism both with exogenously administered hydrocortisone in the adrenalectomized animal (Fig. 4) and with circulating endogenous corticosterone in the normal animal (Fig. 3), progesterone was found to be entirely without effect upon the induction of TAT by dexamethasone.

Discussion. The initial unexpected finding that progesterone (an antiglucocorticoid hormone virtually devoid of glucocorticoid activity *in vitro* (2)) was a potent inducer of TAT in normal rats suggested several possibilities: (i) that progesterone, *in vivo*, might indeed possess intrinsic glucocorticoid activity; (ii) that the observed rise in enzyme activity in response to progesterone was not in fact due to an increased rate of TAT synthesis but rather to a decreased rate of enzyme degradation; or (iii) that the observed induction of TAT, rather than reflecting a direct action of progesterone itself, was instead an indirect effect of the exogenous hormone mediated by endogenous glucocorticoid. Such latter (indirect) effects might, for example, include a nonspecific (e.g., stress-mediated) secretion of corticosterone in response to progesterone (a possibility which would be of only trivial interest), or instead a true potentiation of enzyme induction in response to preexisting circulating endogenous glucocorticoid. The possibility that progesterone itself or one of its metabolites might possess intrinsic glucocorticoid activity *in vivo* was tested and excluded by experiments in adrenalectomized animals. In these latter animals progesterone was nevertheless shown to markedly potentiate the effects of a defined dose of exogenous glucocorticoid, suggesting that the observed induction of TAT by progesterone alone in the intact animal might similarly be mediated by a potentiation of the effects of a preexisting level of endogenous glucocorticoid rather than by a progesterone-induced stimulation of endogenous glucocorticoid secretion. The possibility that the synergistic effect of progesterone was attributable to a decreased rate of enzyme degradation was

not only argued to be implausible from a priori considerations, but was further rendered unlikely by the observation that the synergistic effect of progesterone upon TAT induction disappeared at high doses of exogenous glucocorticoid.

Two additional mechanisms which might have accounted for a true potentiation of glucocorticoid-induced TAT synthesis by progesterone, and which would have been consistent with the preceding experimental observations, were the following: (i) The synergistic effect of progesterone in the presence of either endogenous corticosterone or low doses of exogenous hydrocortisone might be attributable to a reduction in the rate of metabolic clearance of glucocorticoid (e.g., by competition of progesterone for a pathway normally resulting in glucocorticoid inactivation) and a consequent rise in total plasma glucocorticoid concentration; or (ii) more interestingly, progesterone, a hormone with known high affinity for transcortin (11), might compete with corticosterone and hydrocortisone for glucocorticoid binding sites on the carrier protein and thereby induce a rise in the concentration of free (unbound) glucocorticoid. The first explanation would appear to be unlikely to account for the rapid induction of TAT in intact animals by progesterone alone since the endogenous secretory rate for corticosterone is on the order of only about 20 μg/100 g body wt per hour (12, 13), and even in the absence of negative feedback mechanisms an inhibition of its degradation would not be expected to lead to a large elevation in plasma level within the relatively short duration of an experimental period. (The time-course of TAT induction is such that TAT levels 4 hr after the administration of an inducing agent are largely dependent upon glucocorticoid-initiated transcriptional events occurring during the first 2 hr (14).) The second explanation, in addition to being of intrinsically greater interest, was directly testable, for it led to the specific prediction that the phenomenon of potentiation observed in the instance of both exogenous hydrocortisone and endogenous corticosterone should be absent in the instance of dexamethasone, a glucocorticoid which, in contrast, does not bind to transcortin (2, 15). The demonstrated absence of potentiation in the instance of dexamethasone not only confirmed this prediction, but further showed that the potentiation observed in the instance of hydrocortisone was not to be ascribed to some heretofore undescribed "permissive" action of progestins on the expression of glucocorticoid effects.

Despite the numerous examples which can be drawn from different *in vitro* systems (1-8), progesterone—a steroid with clearly defined antiglucocorticoid properties *in vitro* (2)—has yet to be demonstrated to have an antiglucocorticoid action in any *in vivo* system. The possible pitfalls in the interpretation of such experiments are illustrated by the present set of observations which show that the administration of progesterone can, in fact, potentiate the effects of both endogenously secreted and exogenously administered natural glucocorticoids in the induction of TAT. That the potentiating effect of progesterone on glucocorticoid-induced enzyme synthesis is not due to any peripheral synergism at the cellular level, but rather is attributable to a competition for glucocorticoid binding sites on transcortin, is supported by the fact that transcortin is known to have a higher affinity for progesterone than it has for either hydrocortisone or corticosterone (11), and by the demonstration that induction in response to dexamethasone is entirely unaffected by progesterone administration. Since progesterone competes potently with both corticosterone and hydrocortisone for binding to transcortin, whereas in contrast it competes only weakly with either of these hormones for the cytosolic glucocorticoid receptor (2, 16), its effects on glucocorticoid-induced phenomena *in vivo* may be expected to be complex and at times "paradoxical." In contrast to the readily demonstrable antiglucocorticoid effects of progesterone *in vitro*, effects observed in the intact animal may reflect a balance between true antiglucocorticoid effects of the hormone at the cytosol receptor level and potentially obscuring "glucocorticomimetic" effects due to concomitant rises in free glucocorticoid levels brought about by displacment of endogenous glucocorticoid from its major storage site in transcortin.

Summary. Progesterone, a steroid hormone with well-defined antiglucocorticoid proper-

vitro, is shown, paradoxically, to be a inducer of hepatic tyrosine amino-ase in normal but not in adrenalec-l rats. It is further shown that proges- in adrenalectomized animals, mark-tentiates the induction of TAT by low f hydrocortisone, but that this syner-isappears at high doses of the latter ıe. In contrast, there is no synergism n progesterone and the synthetic ste-xamethasone, which, unlike both hy-isone and the natural endogenous glu-coid corticosterone, does not bind to rtin. These observations suggest that ıe induction of TAT in the normal , as well as the potentiation of the ıg effects of low doses of exogenous ortisone in the adrenalectomized ani-e attributable to the displacement by erone of endogenous corticosterone ogenous hydrocortisone, respectively, anscortin and the consequent rise in tration of free circulating glucocorti- Although progesterone competes y with natural glucocorticoids for rtin, it does so only weakly for the ic glucocorticoid receptor. In contrast ırely antiglucocorticoid effects *in vitro*, cts of progesterone in the intact ani-ıy thus reflect a balance between true cocorticoid effects at the cytosol recep-l and other "glucocorticomimetic" ef-ıe to a rise in free glucocorticoid level g from the displacement of endoge-lucocorticoid from plasma binding

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Regional Differences in the Binding Affinities of β-Adrenergic Receptors in the Canine Kidney Cortex (40661)[1]

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The coupling of β-adrenergic neurotransmitters to membrane-bound receptors results in the activation of cyclic adenosine monophosphate (cAMP) which in turn leads to specific physiologic responses (1). In the mammalian kidney, the stimulation of β-adrenergic receptors may be manifested by increases in renal blood flow (2), sodium and water excretion (3), and/or renin release (4). All of these responses parallel the actions of intrarenally administered cAMP or its analogs (5–7).

Recent studies have suggested a neurogenic role in renal tubular sodium reabsorption (8). Direct contact of adrenergic nerve terminals with basement membranes of proximal and distal tubules has been demonstrated in several species (8, 9). Renal denervation decreases and renal nerve stimulation increases proximal tubular sodium reabsorption (10, 11). Other studies have demonstrated in the dog that intrarenal administration of either isoproterenol or dibutyryl cAMP can decrease proximal tubular sodium reabsorption without changes in renal blood flow (3, 5). Chabardes *et al.* (12) have reported that in the rat β-adrenergic stimulation results in increases in adenylate cyclase activity in segments of the distal tubule, suggesting a further role of the adrenergic system in renal tubular sodium transport.

The kidney is structurally and functionally heterogenous (13). Nephrons from the superficial cortex have been shown to differ from inner cortical nephrons in glomerular size, plasma flow, filtration, and sodium reabsorption (13). The degree of adenylate cyclase activation produced by hormones may vary depending upon the regional location of these nephrons (14). In addition, differences in adrenergic innervation and norepinephrine content in the inner and outer regions of the kidney have been noted (15, 16). Therefore, when studying tubular adrenergic receptors, it becomes important to assess membranes derived from both the inner and outer areas of the kidney cortex.

The advent of highly specific radioligands for direct binding studies of β-adrenergic receptors (17) has made possible the quantification of these receptors in a variety of tissue sources (18–20). Utilizing the radiolabeled β-adrenergic antagonist, [^{3}H]dihydroalprenolol ([^{3}H]DHA), studies were undertaken to characterize β-adrenergic receptors in tubular tissue obtained from two different regions of the canine kidney cortex. The results indicate that β-adrenergic receptors within the renal cortex exhibit distinct regional differences in their affinities for dihydroalprenolol.

Materials and methods. Adult mongrel dogs kept on a standard laboratory diet (Purina Chow) were sacrificed by intravenous administration of sodium pentobarbital. Both kidneys were immediately excised, decapsulated, and placed into an ice-cooled (4°) buffer consisting of 0.25 *M* sucrose, 10 m*M* Tris–Cl, and 1 m*M* EDTA adjusted to a pH of 7.5. The kidneys were sagitally sectioned and the cortex divided into an outer 2/3 and inner 1/3 segments (based on wet weight) by methods previously described from this laboratory (21). Renal plasma membranes from each area were subsequently isolated by differential centrifugation techniques according to the procedure of Fitzpatrick *et al.* (22). The final pellet (containing primarily tubular membranes) was resuspended in an incubation buffer consisting of 75 m*M* Tris–Cl and 10 m*M* $MgCl_2$ (pH 7.5). β-Adrenergic receptors in renal plasma membranes were subsequently assayed using the ligand [^{3}H]DHA according to the method of Lefkowitz (23)

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vacuum filtration technique. All in-
ıs were conducted in 150 μl of incu-
ıuffer (final volume) at 23°. The tem-
: of 23° was chosen because at 37°
eous dissociation of the ligand from
nbrane occurred within 5 min. Incu-
ime was 7 min except for the associ-
issociation studies.
ıiation and dissociation kinetics for
ıd outer cortical membranes (approx-
1.0 mg protein) were performed using
[^{3}H]DHA. Following a 2-min equili-
period association studies were
by the addition of the ligand to the
ion mixture and assessed at periodic
s for at least 15 min. Dissociation of
nd from the receptor was initiated by
lition of l-propranolol (10^{-4} *M*) to
ınes previously incubated with [^{3}H]-
ır 7 min. The dissociation was assessed
dic intervals for at least 15 min.
ıity of ligand binding to renal plasma
ınes was assessed in a similar fashion
bating [^{3}H]DHA (30 n*M*) in the pres-
varying concentrations of membrane

)HA binding curves for each tissue
:termined using a fixed amount of
ıne protein (approximately 1.5 mg)
ed with varying concentrations of the
and. In each experiment, nonspecific
of [^{3}H]DHA (approximately 20% of
l binding) was determined from con-
incubations performed in the pres-
f l-propranolol (100 μ*M*). Specific
was assessed as the difference be-
he total and the nonspecific binding.
ır occupancies (R_o) and dissociation
ts ($-K_d$) were calculated from analy-
catchard plots.
petition experiments were also per-
in which varying concentrations of
ıists were added to the incubation
: as indicated. Each assay was per-
in triplicate. Protein concentrations
easured by the Lowry method (24).
ıtions of canine heart plasma mem-
were assayed as a reference tissue.
obtained for $-K_d$ and R_o using the
ıembranes are in agreement with pre-
:ports (18) (Table I).
'ts. At 23° the association of [^{3}H]DHA
to renal plasma membranes was
reaching equilibrium within 5 min (Fig. 1). This equilibrium was maintained for at least 15 min. Following the addition of l-propranolol to an equilibrated mixture of membrane and [^{3}H]DHA, dissociation of bound ligand was maximum in 3 min for outer cortical membranes and 5 min for inner cortical membranes.

The linearity of [^{3}H]DHA binding to renal plasma membranes at various protein concentrations is shown in Fig. 2. This figure reveals a difference in the binding capacities of the two cortical regions. At each protein concentration the inner cortex exhibited a greater ability to bind the radioligand than the outer cortex.

Specific binding of [^{3}H]DHA to partially purified renal plasma membranes was saturable for both the inner and the outer cortical areas. Scatchard plot analysis of the binding data showed two distinctly different linear

TABLE I. TOTAL RECEPTOR OCCUPANCY (R_o) AND DISSOCIATION CONSTANTS ($-K_d$) OF [^{3}H]DHA BINDING TO PARTIALLY PURIFIED PLASMA MEMBRANES FROM HEART AND FROM THE INNER AND OUTER CORTICAL REGIONS OF THE KIDNEY[a]

Tissue	*N*	R_o (pmole/mg protein)	$-K_d$ n*M*
Outer cortex	(4)	0.5216 ±0.0067	80.52 ±3.88
Inner cortex	(4)	0.4558 ±0.0319	37.33 ±1.55*
Heart	(7)	0.3573 ±0.0196	13.27 ±1.65

[a] The results are expressed as the mean ± SE. *N* represents the number of samples assayed. Statistical differences between means were assessed using the Student's *t* test (two-tailed test).

* $P < 0.001$ between the outer and the inner cortex.

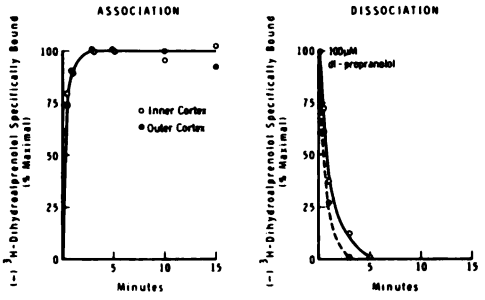

FIG. 1. Association and dissociation kinetics for inner and outer renal cortical membranes. Each point represents the mean of triplicate determinations from two separate assays.

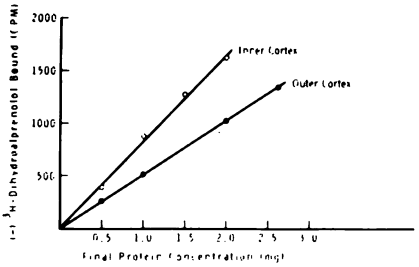

FIG. 2. Protein-dependent binding of [^{3}H]DHA to renal plasma membranes. Each point represents the mean of triplicate determinations from two separate assays. Linear regression analysis of the data points gives a line for the inner cortex as $y = 8.6 + 830x$ ($r = 0.9992$) and for the inner cortex as $y = 6.08 + 506x$ ($r = 0.9998$).

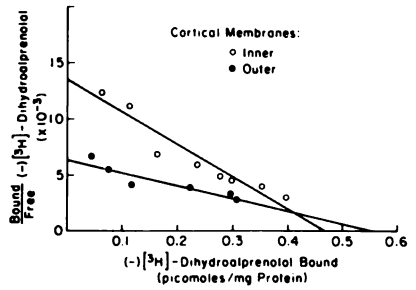

FIG. 3. Scatchard plot of (−)-[^{3}H]dihydroalprenolol binding to renal plasma membranes from the inner and the outer renal cortical areas. Analysis of the data points yields a linear regression line of $y = 0.0133 - 0.0281x$ for the inner cortex ($r = 0.9515$) and $y = 0.0064 - 0.0116x$ for the outer cortex ($r = 0.9243$).

regression lines (Fig. 3). A greater slope (or affinity) was seen for inner cortical than outer cortical membranes ($P < 0.05$). Table I shows the mean values for each cortical region. Although total receptor occupancy was slightly but not significantly greater in outer cortical membranes, the dissociation constant was approximately twofold higher in outer cortical membranes. The greater binding capacity demonstrated for inner cortical membranes therefore is due to the greater affinity of these membranes rather than the number of receptor sites.

Hill coefficients of the binding data for each renal plasma membrane approximated 1, indicating the absence of cooperative binding. Additional studies using this same procedure showed β-adrenergic stereospecificity for both membranes; l-propranolol was approximately 100-fold more effective in displacing [^{3}H]DHA than the D-isomer. Moreover, the α-adrenergic antagonist, phentolamine, was relatively ineffective in displacing the radioligand from either cortical membrane (Fig. 4).

Discussion. There is substantial evidence to suggest a role for β-adrenergic receptors in sodium reabsorption (via cAMP) at different nephron sites (3, 12, 25). Since the kidney has been shown to be heterogenous in terms of adrenergic innervation (16), renin concentrations (26), and nephron function (13), the presence of distinct differences in the binding of [^{3}H]DHA to inner and outer cortical membranes may represent an additional functional difference in regional activities of the kidney.

Investigations by Atlas *et al.* (27) using a fluorescent β-adrenergic antagonist, 9-amino-acridic propranolol, have shown β-adrenergic receptor binding sites in the epithelium of the proximal, distal, and collecting duct portions of the rat nephron as well as in the juxtaglomerular areas. However, these authors did not distinguish the intracortical location of these receptors.

Recently Eliahou *et al.* (28) using a similar membrane preparation technique have characterized [^{3}H]DHA binding sites in rat kidney tissues. Their studies showed a $-K_d$ of 9.3 n*M* and a R_o of 0.0695 pmole/mg of protein in plasma membranes prepared from whole kidneys. In their study, whole kidney homogenate rather than cortical tissue was assayed. Since binding sites appear primarily located in the proximal and distal tubules (27) preparations of cortical tissues should contain a greater concentration of β-adrenergic receptors than whole kidney. Differences between

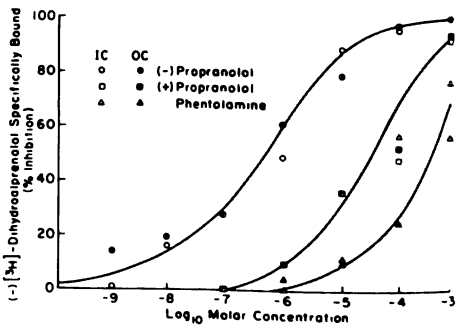

FIG. 4. Stereospecificity of [^{3}H]DHA binding to inner (open circles) and outer (closed symbols) cortical membranes. Each point represents the mean from three assays determined in triplicate.

lings and theirs may also be a reflec-
the incubation temperatures used (23
, or species variations. Although a
in β-receptor response with tempera-
been reported (decreases in temper-
nd to reduce β- and increase α-recep-
is phenomenon occurs mainly at tem-
es below 23° (29). Moreover, these
appear related to changes in cAMP
GMP production rather than receptor
ions (30).

nt evidence has indicated that the kid-
ntains both β_1- and β_2-type subpopu-
of β-adrenergic receptors (31). Burnett
9) have reported that in the lung the
β_1- to β_2-type receptors determines
nity for binding; this raises the possi-
at variations in tubular receptor affin-
erved in different areas of the kidney
nay be due to differences in the rela-
ounts of these β-adrenergic subtypes.
een demonstrated in the brain (32)
number of β-B_1 receptors is regulated
ogenic activity whereas the β-B_2 re-
are apparently independent. If this
ship holds as well in the kidney de-
on of β-receptor subtypes may further
he role of neurogenic activity in so-
absorption.

nary. β-Adrenergic receptors were
erized from plasma membranes ob-
rom two different regions of the ca-
ney cortex by studying their binding
adiolabeled β-adrenergic antagonist
ydroalprenolol. Total receptor occu-
was slightly but not significantly
in outer cortical membranes; the dis-
n constant was approximately twofold
in the outer cortical membranes. A
binding capacity demonstrated for
ortical membranes was due to greater
of the receptors in this area rather
difference in receptor sites. β-Adre-
tereospecificity was demonstrated in
mbranes; l-propranolol was more ef-
in displacing [^{3}H]DHA than D-pro-
l.

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Effect of Altered Copper Metabolism Induced by Mottled Alleles and Diet on Mouse Tyrosinase (40662)[1]

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In mice, alleles at the mottled locus on the X-chromosome produce dramatic influences on coat color (1, 2). Nonagouti mice homozygous for the wild-type (+) allele are black in color, whereas male mice hemizygous for tortoise (*To*), dappled (Mo^{dp}), brindled (Mo^{br}), viable brindled (Mo^{vbr}), or blotchy (Mo^{blo}) are gray (3). Females heterozygous for the mutant alleles are a mosaic of light and dark areas of coat color owing to random inactivation of the X-chromosome (3).

In addition to influences on pigmentation, alleles at the mottled locus are also known to influence behavior, longevity, activity of copper-containing enzymes, copper levels within various tissues, and development of connective tissues (4, 5). Collectively, observations to date on mottled mice suggest that the primary defect resulting in the complications of the "mottled syndrome," as in Menkes' kinky hair syndrome in man (6), is associated with faulty absorption, transport, or binding of copper (5, 7). The alleles at the mottled locus differ in their influences on the activity of copper-containing enzymes such as cytochrome c oxidase, lysyl oxidase, and ceruloplasmin (5, 8, 9). All mutant alleles, however, reduce melanin levels in the pelage, suggesting some common action on the copper-containing enzyme tyrosinase. The presence of coat color mosaicism in female mice heterozygous for mutant mottled alleles indicates that significant gene action occurs at the level of the melanocyte (4). Based on the observation that the intraperitoneal injection of copper results in darker hair color in brindled (Mo^{br}) male mice (10), it would appear that tyrosinase activity is increased when sufficient copper is administered in an appropriate fashion. As yet, however, no direct examination has been made of the form and function of tyrosinase within the melanocytes of mottled mice. The present study was carried out to determine the nature of action of alleles at the mottled locus on tyrosinase activity and the degree to which dietarily induced copper deficiency might mimic the genetic influence. Attention has been given not only to the effects of mottled alleles and dietarily induced copper deficiency on overall tyrosinase activity but also to those on the multiple forms of tyrosinase separable by polyacrylamide gel electrophoresis (PAGE). The latter observations were particularly instructive for the PAGE-separable varieties of tyrosinase are currently considered to represent stages in the developmental sequence leading to mature tyrosinase (11). Depending on genic constitution, a maximum of three soluble tyrosinases (T_1, T_{2-3}) are demonstrable in mouse "hair bulb" extracts subjected to PAGE (12).

Materials and methods. The following strains of mice were used: C57BL/6J, C57BL/6J-Mo^{blo}, C3H/HeJ-Mo^{br}, SWR/J, DBL/Wq, C57BL/Ha-*e*, PLN/Wq, PBR/St, LT/Ch, L/St, and Y/Wi. Hair growth was induced by plucking the quiescent (telogen) hairs from the dorsa of 8-week-old male and female mice. Hair bulbs were obtained on Day 10 of the ensuing hair growth cycle by methods fully described previously (13, 14). Hair bulbs were also obtained during the first spontaneous hair growth cycle from 7-day-old brindled C3H/HeJ-Mo^{br} males and their male agouti littermates.

Copper deficiency was induced in C57BL/6J black mice by placing weanlings on a copper-deficient diet (ICN Pharmaceuticals, Inc.). In some cases penicillamine (0.075%) and pyridoxine (0.02%) were added to the diet. The mice were maintained on the diet for approximately 4 weeks prior to plucking

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niddorsal hair and for an additional
; thereafter. The period of treatment
fficient to reduce the pigmentation
tense black to light brown in the hairs
ı. Hair bulbs were obtained on Day
.e hair cycle induced by plucking.

hair-bulb sample was homogenized
tter–Elvehjem all glass homogenizer
:d 0.25 *M* sucrose (wet wt/vol, 0.1 g/
ł centrifuged at 35,000*g* for 30 min at
resulting supernatant was subdivided
ıe aliquot being supplemented with
by addition of 3×10^{-3} *M* $CuSO_4$ in
sucrose (final concentration of $CuSO_4$
0^{-4} *M*) and the other diluted with the
)lume of 0.25 *M* sucrose. The aliquots
en subjected to PAGE according to
hod of Davis (15) with the following
ations: Distilled water was substituted
ıtion F, the sample gel was omitted,
yme preparation was layered directly
)f the spacer gel, and the bath buffer
ith the stock concentration. The run-
ne for the electrophoresis was 30–40
3–5 mA/tube. Following electropho-
e gels were neutralized in 1.0 *M* phos-
uffer (pH 6.8) for 30 min. The multi-
ns of tyrosinase were visualized by
ion of the gels in 0.1 *M* phosphate
pH 6.8) containing 0.15% L-3,4-dihy-
ıenylalanine (dopa) for 2 hr at 37°.
erve the tyrosinase patterns, gels were
n 7.5% acetic acid. The gel electro-
ams were assigned grades ranging
to ++++ for color intensity of the
ıelanin bands. The visual comparisons
ed well with objective densitometric
:ments (14).

: aliquots of the 35,000*g* supernatants
sayed for the tyrosine hydroxylating
/ of tyrosinase according to the
of Pomerantz (16). The reaction mix-
ıtained 0.15 μmole L-tyrosine, 0.0025
[3,5-^{3}H] tyrosine (New England Nu-
nd 0.15 μmole dopa in each 1.25 ml
of 0.1 *M* phosphate buffer (pH 6.8).
nl of this reaction mixture a 1-ml
of the 35,000*g* supernatant was added;
ıe PAGE, copper was either omitted
:nt in the supernatant in a concentra-
3×10^{-4} *M*. When determining the
attributable to background, 0.25 *M*
was substituted for the supernatant in
the reaction vessel.

Results. Measurements by the Pomerantz radioassay with tyrosine as substrate revealed that the Mo^{br} allele in the hemizygous (Mo^{br}/y) and heterozygous ($Mo^{br}/+$) condition markedly reduced tyrosinase activity in hair-bulb supernatants containing soluble tyrosinase and particulate tyrosinase of the microsomal ("small granule") fraction (Table I). Addition of exogenous copper to the brindled hair-bulb supernatants greatly increased tyrosinase activity while having little effect on control samples (Table I). The tyrosinase activity of copper-supplemented hair-bulb supernatants of brindled mice never exceeded that of the copper-supplemented controls.

Polyacrylamide gel (dopa-incubated) electropherograms revealed that the mottled alleles, Mo^{br} and Mo^{blo}, markedly reduced the activity of the T_1 and T_{2-3} varieties of soluble tyrosinase although they had no effect on their mobilities (Fig. 1 and Table II). T_{2-3} designates a double band of activity, the discreteness of which remains uncertain (11, 12). The addition of copper to 35,000*g* supernatants of brindled and blotchy hair follicles resulted in a marked increase in T_1 activity but not of T_{2-3} judged by the intensity of melanin deposited in dopa-incubated electropherograms. In fact, T_{2-3} activity appeared to be reduced in these cases. No comparable effects were found with hair-bulb supernatants from C57BL/6J control mice (Fig. 1 and Table II). It is noteworthy that the T_1 activity of the copper-supplemented mottled

TABLE I. EFFECT OF COPPER SUPPLEMENTATION ON THE TYROSINASE ACTIVITY OF FOLLICULAR MELANOCYTES OF BLACK AND BRINDLED MICE

	cpm (^{3}HOH)[a]	
	Without copper	Copper added
Black ♀ (+/+) C57BL/6J adult control	2671	2940
Brindled ♀ ($Mo^{br}/+$) adult	230	1500
Brindled ♂ (Mo^{br}/y) 7 day old	0	316
Black Agouti ♂ (+/+) 7 day old control	781	779

[a] Pomerantz radioassay. Each sample consisted of 1 ml of supernatant from the pooled hair bulbs of 3–4 adult mice or 11–14 neonatal mice (w/v = 0.2 g/ml in adults and 0.1 g/ml in neonates). Experimental and control samples were run simultaneously. In each case a single sample was counted after 1 hr incubation.

supernatants decisively exceeded the T_1 activity of the copper-supplemented control supernatants.

The addition of copper to 35,000*g* hair-bulb supernatants from mice of a variety of coat colors resulting from action of genes independent of the mottled locus failed to increase tyrosinase activity as measured by the intensity of melanin synthesized within dopa-incubated electropherograms (Table III). In no case did exogenous copper result in the appearance of extra bands of tyrosinase or of tyrosinase bands where they were characteristically absent for a given genotype (17).

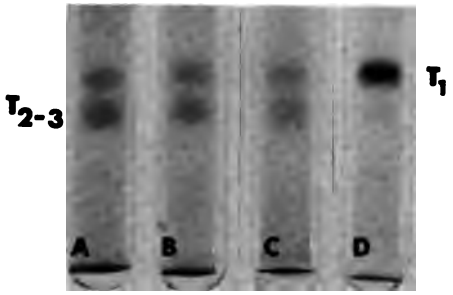

FIG. 1. Activity patterns of tyrosinase from follicular melanocytes of black (A and B) and brindled (C and D) female mice: A and C no copper supplementation; B and D copper added prior to electrophoresis.

The tyrosinase from C57BL/6J mice dietarily deprived of copper closely approximated that of the mottled mice in its properties. Tyrosinase activity as measured by the Pomerantz radioassay was markedly reduced in hair-bulb supernatants from mice with dietary copper deficiency and greatly increased when copper was added to the supernatants (Table IV). In addition, dopa-incubated electropherograms demonstrated that both the T_1 and T_{2-3} varieties of tyrosinase were reduced in activity by the copper deficiency and that the activity of T_1 but not of T_{2-3} could be increased by the addition of exogenous copper to the supernatants prior to electrophoresis (Table IV).

Discussion. The results of this study indicate that the uniform hypopigmentation of male mice hemizygous for mottled alleles and the patchy depigmentation of heterozygous females results from reduced tyrosinase activity within affected melanocytes. The reduced

TABLE II. EFFECT OF COPPER SUPPLEMENTATION *IN VITRO* ON THE ACTIVITY OF SOLUBLE TYROSINASES FROM MOTTLED AND BLACK MICE

	D-PAGE[a]			
	Without copper		Copper added	
	T_1	T_{2-3}	T_1	T_{2-3}
Black ♂, ♀ (+/+) C57BL/6J adult	+++	+++	+++	+++
Brindled ♀ (Mo^{br}/+) adult	+	+	++++	<+
Blotchy ♂ (Mo^{blo}/*y*) adult	+	<+	++++	<+

[a] D-PAGE = DOPA-incubated polyacrylamide gel electropherograms. <+ = perceptible but marginal activity.

TABLE III. EFFECT OF COAT COLOR GENES AND COPPER SUPPLEMENTATION *IN VITRO* ON THE ACTIVITY OF SOLUBLE TYROSINASES

Genotype							D-PAGE[a] relative activity with or without added copper	
a	b	c	d	e	ln	p	T_1	T_{2-3}
a/a	B/B	C/C	D/D	E/E	Ln/Ln	P/P	+++	+++
a/a		c/c					0	0
a/a			d/d				+++	+
a/a				e/e			++	0
a/a					ln/ln	p/p	++	0
a/a	b/b					p/p	+++	0
a/a	B^{lt}/B^{lt}						++	++
A^w/A^w		c^{ch}/c^{ch}					+++	+
A^y/A							+	0

[a] DOPA-incubated polyacrylamide gel electropherograms.

V. Effect of Copper Supplementation *in Vitro* on the Tyrosinase Activity of Hair-Bulb Supernatants of Normal and Copper-Deficient C57BL/6J (Black) Mice

Treatment	PRA[a] cpm (^{3}HOH)		D-PAGE[b] relative tyrosinase activity			
	No copper	Copper added	No copper		Copper added	
			T_1	T_{2-3}	T_1	T_{2-3}
ete diet	430	587	+++	+++	+++	+++
r-deficient diet	36	393	++	+	++++	+

ıtz radioassay.
ıcubated electropherograms.

activity of mottled mice appears to bstantially from restrictions in the f copper available for combination proteinaceous apoenzyme to pro- ctional tyrosinase. Dopa-incubated erograms show that exogenous cop- ıced soluble T_1 activity of mottled preparations to above control val- sinase activity toward tyrosine as a is greatly increased when copper is nottled hair-bulb supernatants con- luble tyrosinase and particle-bound al tyrosinase, but its activity re- ow that of the controls.

ively, the results suggest that in mot- considerable amounts of tyrosinase re synthesized but remain inactive an inadequate supply of copper e melanocyte. The essentially iden- ıerties of mottled and dietarily in- pper-deficient tyrosinase prepara- port this conclusion. The alleles of ed locus apparently influence tyro- tivity and, as a consequence, pig- ı by acting on melanocytes to reduce vels below those necessary to pro- ırmal complement of functional ty- nolecules. The patchy light and dark male mice heterozygous for mottled arly indicates that the genes act at of the melanocytes. Applying the ıothesis (18), the dark areas are as- be populated by melanocytes with X-chromosome bearing the wild- e. These melanocytes are expected e more melanin than those in the s where the active X bears the mot- . In reality, it is possible that the ıelanocytes of at least some mottled otes synthesize somewhat less pig- n those of wild-type mice owing to intestinal absorption, transport, or binding of copper. That this is the case is suggested by the increased pigmentation of hairs grown after intraperitoneal injections of copper in male brindled mice (10).

The electrophoretic studies reported here suggest that although T_1 and T_{2-3} are reduced owing to the action of the mottled alleles and of dietarily induced copper deficiency, only T_1 is increased in activity by copper supplementation. Apparently, the bulk of the copper-deficient apoenzyme is of the T_1 variety or converted to it by addition of copper. Evidence suggests that T_1 is a glycoprotein possibly derived from T_3 by addition of sialic acid and neutral sugars (11). It is not clear why T_3 should be saturated with copper whereas its putative derivative T_1 is not. Preliminary electron microscopic studies suggest that melanogenesis within individual melanosomes is reduced, indicating that they are deficient in tyrosinase or endowed with some copper-deficient tyrosinase.

At present it is not known whether T_1 tyrosinase apoenzyme migrates with the complete T_1 metalloprotein or is located at some other site in the gel. Future studies will isolate T_1 from the gels to determine whether addition of exogenous copper enhances its activity on reelectrophoresis and treatment with dopa reagent.

The present results suggest that the alleles of the mottled locus regulate melanogenesis by influencing the availability of copper at the level of the melanocyte perhaps by an action on copper transport or binding. In view of the multiple defects associated with the mottled alleles, it has been proposed that they represent a series of overlapping deletions with a common defect in pigmentation (9). A similar conclusion has been reached about the radiation-induced lethal alleles of the albino locus (19). Thus, the true nature of

the mottled locus remains to be resolved as does the simplicity or complexity of its primary action. Elucidation of this important point in mottled mice may help clarify the molecular genetics underlying the comparable condition of Menkes' kinky hair syndrome in humans (6, 20).

Summary. An analysis of tyrosinase activity in extracts of hair bulbs from mice bearing alleles at the mottled locus, Mo^{br} (brindled), and Mo^{blo} (blotchy), indicates that reduced melanogenesis in mottled mice is associated with restricted availability of copper. Although tyrosinase activity in 35,000*g* supernatants of hair-bulb homogenates of mottled mice is reduced compared to that of nonmottled controls, addition of copper to the former but not the latter supernatants markedly increases tyrosinase activity as measured in polyacrylamide gel electropherograms (dopa as substrate) or by the Pomerantz radioassay (tyrosine as substrate). Comparable results were obtained when tyrosinase from dietarily copper-deficient hypopigmented mice was assayed by either method. In the electropherograms of dietarily and genetically copper-deficient hair-bulb supernatants, the enhancement of activity is selectively restricted to the T_1 variety of tyrosinase. It is concluded that alleles at the mottled locus influence tyrosinase and consequently melanin pigmentation by acting on melanocytes to reduce copper levels below those necessary to produce a normal complement of functional tyrosinase molecules.

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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 269–274 (1979)

genic Amine Involvement in Pyrogenicity and Enhancement of Lethal Endotoxin Shock by Group A Streptococcal Pyrogenic Exotoxin (40663)

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up A streptococcal pyrogenic exotoxin type C produces fever in rabbits by stimulation of the hypothalamic tem- re control center, rather than indirectly h leukocyte endogenous pyrogen (1). orted for other pyrogens (2–4), fever ction by SPE C was inhibited (5) by the glandin synthetase inhibitor salicylate ggesting toxin pyrogenicity depends release of a prostaglandin-like mole- Other mediators of SPE fever have not lefined.

biogenic amines norepinephrine and nin previously were shown to modulate er response of rabbits to pyrogens (7– Whereas rabbits pretreated with α- l tyrosine to deplete norepinephrine showed depressed fever responses after nge with pyrogens (8, 10), intraventric- dministration of norepinephrine re- in fever (7, 11). These studies indicated orepinephrine had a positive effect on production in the rabbit. It was shown r that α-adrenergic stimulation by nor- hrine was necessary for pyrogenicity 12).

letion of serotonin stores using *p*-chlo- ylalanine in rabbits resulted in accen- fever responses after challenge with ns (8), and intraventricular injection otonin depressed fever responses (7). ata suggest serotonin exerted a negative on pyrogenicity.

ddition to production of fever, group ptococcal pyrogenic exotoxins have other biological properties (13–21), in- g capacity to enhance host susceptibil- lethal shock by endotoxin (13) or strep- O (14). The enhancement phenome- as not prevented by treatment of rab- ith the antipyretics salicylate, indo- cin, or cortisone (5). Later it was shown nlike pyrogenicity, enhancement was roperty requiring an SPE effect on the (1).

This study was designed to test the involvement of biogenic amines and α- versus β-receptors in the pyrogenicity of SPE C. Also, the effects of the same agents on the capacity of SPE C to enhance lethal endotoxin shock were examined.

Materials and methods. Purified preparations of SPE C, containing 25% pyrogen-free hyaluronic acid as a stabilizing agent, were made as described previously (1, 13, 22). The minimum dose of toxin given i.v. to produce an average fever response of 0.5° at 4-hr postinjection (MPD-4) was approximately 1 μg/kg rabbit body wt; the dose of toxin used was 20 MPD-4/kg i.v. Toxin concentrations were determined using the microbiuret protein assay (23) with bovine serum albumin (Pentex, Miles Laboratories, Inc., Kankakee, Ill.) serving as the standard.

Biological assays. Solutions of SPE were prepared in sterile pyrogen-free phosphate-buffered saline (PBS 0.005 *M* phosphate buffer, pH 7.0, plus 0.15 *M* NaCl) for all biological assays. American–Dutch belted rabbits 1.0–1.5 kg were used for assays of SPE pyrogenicity and capacity of SPE to enhance lethal endotoxin shock (13). Depending on the day, starting temperatures of the rabbits ranged from 37 to 38.5° but differed by less than 0.5° for individual experiments. Unless otherwise stated, enhanced susceptibility to lethal endotoxin shock was measured by giving an i.v. injection of either 25 or 1 μg/kg of endotoxin (*Salmonella typhimurium*) to each rabbit 4 hr after i.v. SPE injection. The 1 μg/kg dose of endotoxin represented sufficient toxin to kill four or five of five rabbits pretreated with SPE C. The higher dose of endotoxin (25 μg/kg) was employed previously (1, 5) as the standard dose for tests of enhancement of susceptibility to lethal shock by SPE C. The LD_{50} of endotoxin for normal rabbits was 535 μg/kg. The LD_{50} of endotoxin for rabbits pretreated with 20 MPD-4/kg SPE C was 0.028 μg/kg.

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Drugs. *p*-Chlorophenylalanine (PCPA, Aldrich Chemical Co., Inc., Milwaukee, Wis., 300 mg/kg) and α-methyl tyrosine (αMT, Sigma Chemical Co., St. Louis, Mo., 100 mg/kg) were administered i.p. to rabbits as suspensions in PBS 12 hr before toxin treatment. Norepinephrine (Levophed bitartrate, Winthrop Laboratories, N.Y.) and propranolol (Inderal, Ayerst Laboratories, N.Y.) were diluted according to the manufacturers' specifications to 50 μg/0.1 ml. Serotonin (Sigma, 50 μg/0.1 ml) and isoproterenol (Sigma, 50 μg/0.1 ml) were prepared in PBS. Intracisternal (i.c.) injections of these drugs into rabbits were made into the cisterna magna with syringes equipped with 27 gauge by 0.5-in. needles; the volume injected i.c. was 0.1 ml into each rabbit. Phenoxybenzamine (Dibenzyline, Smith, Kline, and French Laboratories, Philadelphia, Pa.) was prepared in PBS and 1 mg/kg injected intravenously. To test the ability of phenoxybenzamine (PBA) alone to suppress fever and prevent enhanced susceptibility to lethal shock, 1 mg/kg was given to rabbits i.v. 1 hr before SPE C and 5 hr before endotoxin. Capacity of PBA and fluid replacement to alter enhancement was assessed using the following procedures: Rabbits given SPE C were treated with PBA (2 mg/kg) 1 hr before receiving endotoxin (1 μg/kg). Immediately after administration of endotoxin and every 15 min for a total of seven injections, each rabbit received i.v. 10 ml of 5% dextrose in pyrogen-free distilled water. Controls consisted of: Rabbits treated with SPE and endotoxin; SPE, PBA, and endotoxin; and SPE, endotoxin, and fluid.

Statistical analyses. The difference in mean fever responses between two groups was evaluated at the 4-hr time point or 1 hr after drug injection, using Student's *t* test analysis. The variability in fever responses is indicated by ± SEM. Statistical analyses were performed only at the 4-hr time point or 1 hr after injection of drug.

Results. Rabbits pretreated with the tyrosine hydroxylase inhibitor αMT showed depressed 4-hr fever responses after challenge with SPE C (Fig. 1) compared to control animals which received PBS and toxin ($P < 0.01$). However, control and experimental rabbits showed comparable enhanced susceptibility to lethal endotoxin shock (enhancement) at the doses of toxin used. There was no apparent difference in timing of deaths between groups.

α-Adrenergic blockage (Figs. 1 and 2) using PBA also inhibited the pyrogenicity of SPE C compared to control animals receiving toxin only ($P < 0.01$). However, alone PBA did not prevent enhancement; 5 of 5 animals died in either group after receiving 25 μg/kg endotoxin (Fig. 1). PBA alone did not inhibit enhancement when animals received a lower dose of endotoxin (1 μg/kg); 9 of 10 rabbits died in each group (Fig. 2). Again, there was no apparent difference in timing of deaths between groups for both doses of endotoxin.

It is important to note that separate PBS controls were run for each group of experimental animals presented in Fig. 1. The variability in average fever response between experiments when animals were given PBS and toxin may have resulted for at least two reasons: Different batches of toxin were used for the experiments, and whereas batches of toxin have comparable MPD-4/kg, differences in pyrogenic activity exist. Also, be-

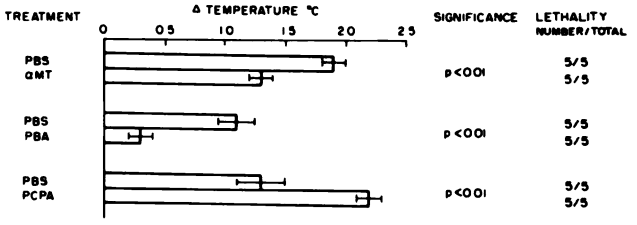

FIG. 1. The effect of pretreatment of rabbits with α-methyl tyrosine (αMT), phenoxybenzamine (PBA), and P-chlorophenylalanine (PCPA) on SPE C fever production and capacity to enhance lethal endotoxin shock. Control animals received phosphate-buffered saline (PBS) followed by SPE C. Shown are the average fever responses of five rabbits in each group 4 hr after SPE C administration i.v. (20 μg/kg). Bars indicate ± 1 SE. The numbers of rabbits in each group that showed enhanced susceptibility to lethal endotoxin shock are shown. Significant differences in the mean 4-hr fever responses between control and experimental groups were assessed using Student's *t* test.

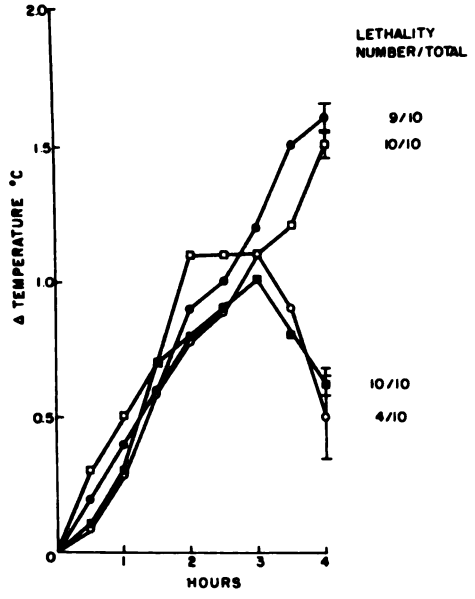

FIG. 2. The effect of phenoxybenzamine (2 mg/kg) on SPE-C-induced fever and together with fluid replacement on capacity to enhance lethal endotoxin shock. Animals (10/group) given: SPE C and endotoxin (●); SPE C, phenoxybenzamine, endotoxin, and fluid replacement (○); SPE C, phenoxybenzamine, and endotoxin (■); SPE C, endotoxin, and fluid replacement (□). Bars indicate ± 1 SE.

cause the experiments were not performed on the same day, differences in animals may have contributed to the variability.

PBA, given in combination with fluid replacement, was partially effective in reversing the course of the enhancement phenomenon as shown in Fig. 2 (endotoxin dose used was 1 μg/kg). Comparable treatment was effective previously in altering the course of lethal endotoxin shock (24–26). Unlike control animals, 6 of 10 rabbits survived challenge with SPE and endotoxin (1 μg/kg) when treated with PBA and fluid.

Rabbits pretreated with PCPA to deplete serotonin stores showed accentuated fever responses (Fig. 1) after challenge with SPE C ($P < 0.01$), but control and experimental animals exhibited enhanced susceptibility to lethal shock.

Administration of norepinephrine i.c. to rabbits with fevers due to SPE C (Fig. 3) resulted in further heightened responses compared to controls receiving diluent ($P < 0.01$) 1 hr after injection. Again, enhancement of lethal shock was not prevented. Unlike norepinephrine, serotonin administered i.c. (Fig. 4) to rabbits exhibiting fevers due to SPE C resulted in a significant reduction of the average fever response ($P < 0.001$) 1 hr after injection. All animals showed enhanced susceptibility to lethal endotoxin shock; endotoxin was given at the 5-hr time point.

The β-receptor stimulating and blocking compounds, isoproterenol and propranolol, did not affect SPE-induced fevers in rabbits and alone did not prevent enhancement.

Discussion. The data presented in this study suggest SPE C-induced fever in rabbits was positively affected by norepinephrine and negatively affected by serotonin. Furthermore, pyrogenicity depended in part upon stimulation of α-adrenergic receptors. Presumably, these observations could be extended to include the rat and sheep, which have been shown to respond comparably to norepinephrine and serotonin (27, 28). However, the reverse would be expected when studying cats, dogs, or monkeys (27, 28); that

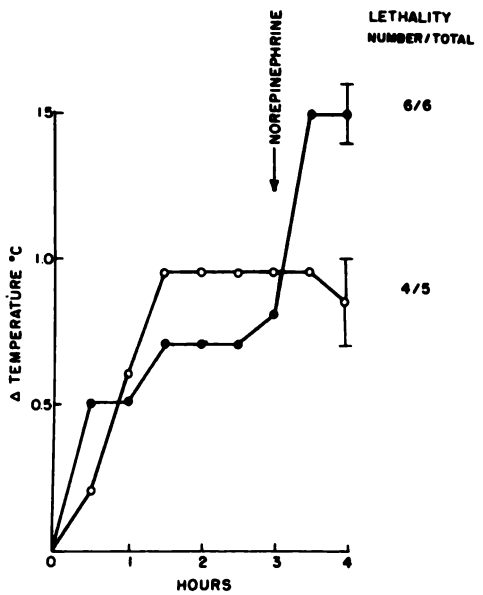

FIG. 3. The effect of intracisternal administration of norepinephrine (50 μg/0.1 ml) on SPE fever production and capacity to enhance lethal endotoxin shock. SPE C (20 μg/kg) was administered i.v. at 0 hr to all rabbits in each group. Norepinephrine (●) or buffer (○) was given 3 hr after SPE C. *Salmonella typhimurium* endotoxin (25 μg/kg) was given i.v. to all animals at the 4-hr time point. Bars indicate ± 1 SE.

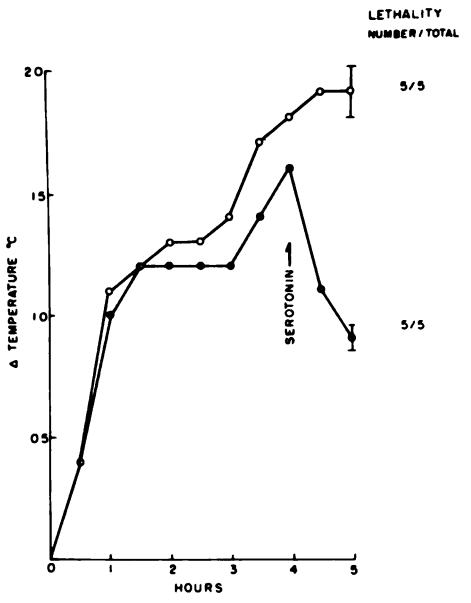

FIG. 4. The effect of intracisternally administered serotonin (50 μg/0.1 ml) on SPE C-induced fever and capacity to enhance lethal endotoxin shock. All animals (five/group) received SPE C (20 μg/kg) at 0 hr followed by serotonin (●) or phosphate-buffered saline (○) at 4 hr. *Salmonella typhimurium* endotoxin (25 μg/kg) was administered i.v. 5 hr after SPE C. Bars indicate ± 1 SE.

is, fever after challenge with serotonin and body cooling after challenge with norepinephrine. The reason for the species variation is not clear.

Researchers have shown that norepinephrine produced fever in rabbits when injected into the hypothalamic area (7, 11). Also, it was suggested that fever production by norepinephrine depended upon α-adrenergic stimulation, since agents such as PBA inhibited fever (9–12). The high concentration of norepinephrine contained in the hypothalamus is consistent with the amine mediating pyrogenicity (29).

Other investigators have implicated norepinephrine as a mediator of endotoxin-induced fever. Intracisternal injection of PBA inhibited endotoxin pyrogenicity (12). Administration of αMT to deplete norepinephrine stores, reduced leukocyte pyrogen fever (8); monoamine oxidase inhibitors potentiated fever (30). The results presented in this study indicate group A streptococcal pyrogenic exotoxin type C produces fever also dependent upon norepinephrine and stimulation of α-adrenergic receptors, although not dependent upon endogenous pyrogen release (1).

In a previous report PCPA (8) was used to deplete stores of serotonin in rabbits (31). Subsequent challenge with leukocyte endogenous pyrogen resulted in accentuated fever responses, indicating the presence of serotonin depressed pyrogenicity. In the present study comparable results were obtained using SPE C to induce fever. Also, administration of serotonin i.c. to rabbits showing fevers resulted in a significant decline in temperatures. As proposed earlier (9), norepinephrine and serotonin may act reciprocally to regulate body temperature. Elevation of norepinephrine relative to serotonin would result in fever whereas the reciprocal would result in body cooling.

It is significant that PBA given i.v. in this study depressed SPE fever production. Previous researchers using a lower dose observed that the drug had to be injected into the hypothalamic area to reduce fever (12). It is possible the higher dose utilized in the present study permitted sufficient PBA to penetrate the blood–brain barrier. Alternatively, SPE C which was shown to alter the permeability of the blood–brain barrier in rabbits (1) may have allowed sufficient PBA to cross.

The treatment of endotoxin shock has been highly controversial (24–26, 32–34). While one approach has been to use steroids and vasopressors (32), a number of investigators have shown endotoxin shock in rabbits responded favorably to PBA plus fluid replacement (24–26). In this study comparable techniques employing PBA plus fluid replacement were partially successful in altering the lethal effect. The partial success in reversing shock may have resulted from a combination of increased cardiac output (26) and blocked catecholamine-induced arteriolar vasoconstriction by PBA (25, 26) together with increased blood pressure after fluid replacement (26).

Based on the observation that PBA and fluid partially reversed the enhancement phenomenon, it would be tempting to speculate that enhancement of lethal endotoxin shock indirectly results from SPE inactivation of the endotoxin detoxification system. Such an effect would allow endotoxin to persist in the

on to produce a typical shock syn-
Other studies are consistent with this
l. It was shown that SPE alters retic-
thelial system (RES) clearance func-
16). Also, a very low dose of endo-
028 μg/kg LD_{50}) is required for le-
fter SPE treatment compared to 535
D_{50} without SPE. A much higher
ndotoxin may be required to produce
the normal animal by overwhelming
detoxification system, thus allowing
amount of endotoxin to persist. An
ve hypothesis is that SPE plus endo-
ıy act synergistically on the same cell
produce lethality, whereas much
oses of endotoxin alone may be re-
› produce the effect. The use of SPE
ination with endotoxin may serve as
for the elucidation of the mechanism
ıcement of lethal shock.

ary. Group A streptococcal pyro-
›toxin type C (SPE C) was shown to
fever which in part depended upon
:phrine and stimulation of α-adrener-
›tors. Intracisternal injection of nor-
rine into rabbits already showing fe-
e to SPE C resulted in further
ed fevers. Pretreatment of animals
ıer α-methyl tyrosine to deplete nor-
rine stores or phenoxybenzamine to
receptors depressed SPE-induced py-
y. Pretreatment of animals with P-
ıenylalanine to deplete serotonin
›centuated fevers due to SPE and
erotonin intracisternally to rabbits
ers resulted in a significant drop in
nperature. This indicated serotonin
ı negative effect on SPE pyrogenicity.
renol and propranolol did not affect
ever production. When used alone,
the drugs prevented the capacity of
to enhance lethal endotoxin shock.
:, phenoxybenzamine in combination
d replacement increased the survival
ıbbits. It is proposed that SPE C may
: endotoxin detoxification system,
wing endotoxin to persist in the cir-
producing shock.

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OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 275–280 (1979)

onse of Erythroid Day 3 Burst-Forming Units to Endotoxin and Erythropoietin (40664)

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sma clot and methyl cellulose cul-
ıtaining erythropoietin (Ep), mor-
ally unidentifiable erythroid progen-
proliferate and differentiate *in vitro*
clusters of erythroblasts (1, 2). By
iguration and time of appearance of
lusters, and by physical and biologi-
:rties of the progenitor cells, murine
l progenitors have been classified
: stages: colony-forming units (CFU-
3 burst-forming units (Day 3 BFU-
Day 8 burst-forming units (Day 8
(1, 3, 4). The CFU-E give rise to
onies of eight or more erythroblasts
of culture with small concentrations
d have a modal sedimentation veloc-
it gravity of 7.0 mm/hr, whereas the
FU-E give rise to clusters of erythro-
onies at 8 days of culture, require
mounts of Ep and have a modal
:ation velocity at unit gravity of 3.8
1, 5). Day 3 BFU-E are intermediate
:haracteristics (5). These three stages
:idered to be part of a continual,
:ed process of erythroid proliferation
erentiation which begins with the
FU-E and proceeds through the Day
: stage and subsequently through the
:tage yielding erythroblasts (6–8).

normal mice were injected with
n, erythropoiesis increased in the
ıd decreased in the marrow (9, 10).
', in plethoric mice, whose Ep levels
low, endotoxin increased the number
ic CFU-E, but not the number of
lasts (11). The fact that increased
of erythroblasts accompanied in-
ıumbers of CFU-E in normal mice
dotoxin, but not in plethoric mice,
d that the presence of Ep enhanced
iation of the splenic CFU-E into
lasts, but that endotoxin did not re-
: presence of Ep to increase the num-
)lenic CFU-E. Because the increase in splenic CFU-E induced by endotoxin was delayed and occurred 24 hr following a single injection, with a maximal increase 72 to 96 hr after injection, Reissmann *et al.* (11) postulated that the increase in splenic CFU-E seen after endotoxin administration was the result of a primary effect of endotoxin on an earlier precursor cell. No further investigation has been reported, however, on the effect of endotoxin on the earlier splenic Day 3 BFU-E. Endotoxin has been identified as a contaminant in Ep preparations (12–14) used by almost all investigators in the study of erythropoiesis, and has been shown to inhibit stimulation of erythropoiesis *in vivo* by these Ep preparations (14). The present study was undertaken to determine the effect of endotoxin on the number of marrow and spleen Day 3 BFU-E with and without the presence of Ep. Using both morphological and biochemical studies of cells from hypertransfused mice, we have found that endotoxin markedly increases the number of splenic Day 3 BFU-E and decreases the number of these cells in the marrow. Endotoxin had little effect on Day 3 BFU-E differentiation. In contrast to the effect of endotoxin, the major effect of Ep was to increase the number of more differentiated erythroid precursor cells, including the CFU-E, in the marrow and spleen. Spleens of plethoric mice given both endotoxin and Ep had increased numbers of the more differentiated erythroid progenitors as observed in mice given Ep alone and had decreased numbers of the earlier erythroid progenitors compared to mice given endotoxin alone.

Materials and methods. Male BALB/c mice from 9 to 12 weeks of age and approximately 20 g were made plethoirc by i.p. injection of 0.5 ml of packed BALB/c erythrocytes on Day 1. On Day 3, mice with microhematocrits ranging from 62 to 65% (normal 48–50%) were selected for treatment on Days 4–7.

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Groups of five or six mice were treated as follows: (i) control—no treatment; (ii) endotoxin treated—daily i.p. injections of 10 μg endotoxin (lipopolysaccharide B *S. enteriditis*, Difco) diluted with Hanks' balanced salt solution (HBSS); (iii) Ep treated—twice daily i.p. injections of 0.5 units sheep Ep (Connaught, Step 3) which had been diluted in HBSS and from which endotoxin had been removed as described by Graber *et al.* (15) with limulus amebocyte lysate (Worthington); (iv) endotoxin + Ep treated—daily injections of 10 μg of endotoxin plus twice daily injections of 0.5 units of limulus-absorbed Ep. On Day 8 hind limb marrow cells were obtained by flushing femoral and tibial marrow cavities with HBSS and then gently passing the cell suspensions through a 27-gauge needle. Spleen cells were obtained by straining sliced spleens through nylon mesh bags from human platelet administration sets. After determining nucleated cell counts with a hemocytometer, the cells were suspended in tissue culture media containing 1.0 unit/ml of limulus-absorbed Ep and incubated at 37° in air plus 5% CO_2.

To quantitate colonies and bursts, cells were cultured in plasma clots consisting of α-medium with 30% heat-inactivated (56° for 60 min) fetal calf serum (Grand Island Biological Co.), 1% deionized bovine serum albumin diluted with phosphate-buffered saline (PBS), 10^{-4} *M* β-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.85% beef embryo extract (GIBCO), and 10% citrated bovine plasma (GIBCO) as described by McLeod *et al.* (16). For determination of CFU-E, suspensions of 5×10^5 nucleated cells/ml were cultured in quintuplicate 0.1-ml aliquots in microtiter wells (Linbro). At 36 hr of incubation, the clots were fixed with 5% glutaraldehyde, stained with benzidine and hematoxylin, and colonies of eight or more benzidine-positive cells were counted, For determination of Day 3 BFU-E, suspensions of 4×10^5 nucleated cells/ml were incubated in triplicate 0.5-ml aliquots in 16-mm-diameter wells (Costar). The clots were fixed and stained at 66 hr of incubation and scored according to the criteria of Gregory (4).

For determination of ^{59}Fe incorporation into heme, cells were incubated in α-medium with 0.8% methyl cellulose, 30% fetal calf serum, 1% bovine serum albumin, 10^{-4} *M* β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin using a slight modification of the method of Iscove and Sieber (2). Cell suspensions of 2×10^6 nucleated cells/ml were incubated in 0.1-ml aliquots in microtiter wells. On each of 7 or 8 consecutive days, five wells were pulsed for 24 hr with 12 μl of a 10 μCi/ml solution of $^{59}FeCl_3$ in iron-poor human plasma using a slight modification of the method of Eliason *et al.* (17). At the end of each 24-hr pulse, the cells were removed from the wells, washed twice with 1 ml of PBS, and lysed with 0.9 ml of 33% Drabkin's solution. After acidification with 0.1 ml of 1 *N* HCl, heme was extracted into an equal volume of cyclohexanone and ^{59}Fe uptake was determined as cpm/culture in an automatic gamma well-type scintillation counter. Greater than 95% of cyclohexanone-extractable ^{59}Fe was found with mouse hemoglobin that was chromatographed on CM-cellulose columns. Statistical significance was determined by Student's *t* test.

Results. Marrow and spleen cells from control mice and mice treated with endotoxin, Ep, or endotoxin + Ep were cultured simultaneously for determination of CFU-E, Day 3 BFU-E, and ^{59}Fe incorporation into heme. The number of erythroid colonies in each group was proportional to Day 1, 0- to 24-hr, ^{59}Fe incorporation, and the number of Day 3 bursts was proportional to Day 3, 48- to 72-hr, ^{59}Fe incorporation (Table I). Because we wished to observe the cumulative kinetic effect of Ep *in vitro* on the erythroid precursor cell compartment, further experiments were performed with daily measurements of ^{59}Fe uptake. By using 1.0 unit/ml of limulus-lysate-absorbed Ep in the medium, marrow and spleen cell cultures from treated and untreated mice had their highest ^{59}Fe incorporation within the first 5 days of incubation (Figs. 1, 2). As several experiments showed low ^{59}Fe incorporation on Days 6–8, subsequent experiments were terminated after 7 days of incubation.

Endotoxin-treated mice had no significant increase over nontreated mice in number of splenic nucleated cells, but they had a very marked increase in Day 3 BFU-E and Days 3–5 ^{59}Fe incorporation (Table I, Fig. 1). A

TABLE I. COMPARISON OF CFU-E AND DAY 1 ^{59}Fe INCORPORATION, PLUS DAY 3 BFU-E AND DAY 3 ^{59}Fe INCORPORATION, OF SPLEEN OR MARROW CELLS FROM HYPERTRANSFUSED MICE TREATED FOR 4 DAYS WITH ENDOTOXIN, ERYTHROPOIETIN (Ep), OR ENDOTOXIN + Ep

Organ Treatment	CFU-E[a] ($\times 10^{-3}$)	Day 1 Heme ^{59}Fe[a] (cpm $\times 10^{-3}$)	Day 3 BFU-E[a] ($\times 10^{-3}$)	Day 3 Heme ^{59}Fe[a] (cpm $\times 10^{-3}$)
Spleen				
None	0.0 ± 0.0	9.0 ± 1.2	11.9 ± 1.6	33.8 ± 4.0
Endotoxin[b]	19.5 ± 5.4	66.6 ± 4.6	93.7 ± 5.2	620.8 ± 12.4
Ep[c]	62.7 ± 5.5	493.9 ± 19.2	39.2 ± 4.7	158.7 ± 9.8
Endotoxin + Ep[d]	189.1 ± 25.9	1054.1 ± 61.5	243.5 ± 24.6	1305.3 ± 100.4
Marrow				
None	3.6 ± 0.4	5.1 ± 0.4	18.9 ± 1.2	60.5 ± 3.1
Endotoxin[b]	0.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	8.6 ± 0.5
Ep[c]	26.4 ± 2.9	45.1 ± 1.5	20.7 ± 1.6	59.7 ± 2.3
Endotoxin + Ep[d]	2.4 ± 0.3	5.0 ± 0.1	5.8 ± 0.5	32.3 ± 1.7

[a] Per spleen or hind limb ±SEM.
[b] 10 μg/day i.p.
[c] 0.5 unit × 2/day i.p.
[d] 10 μg/day + 0.5 units × 2/day i.p.

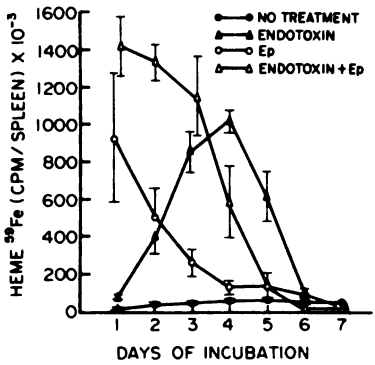

FIG. 1. Daily ^{59}Fe incorporation into heme of cultured spleen cells from hypertransfused mice. After 4 days of treatment, nucleated cell counts × 10^{-6}/spleen were: no treatment, 151.7 ± 7.5; endotoxin, 163.5 ± 2.8; Ep, 192.0 ± 17.0; endotoxin + Ep, 193.0 ± 5.1. Data represent mean ±SEM for six experiments except Ep and endotoxin + Ep groups which represent three experiments.

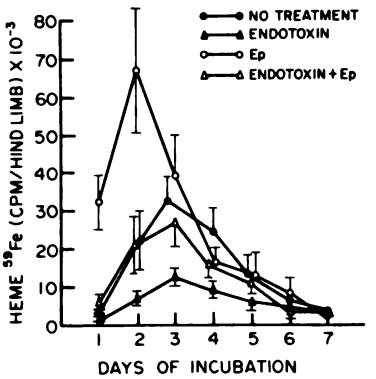

FIG. 2. Daily ^{59}Fe incorporation into heme of cultured marrow cells from hypertransfused mice. After 4 days of treatment, nucleated cell counts × 10^{-6}/hind limb were: no treatment, 18.1 ± 0.8; endotoxin 11.3 ± 0.9; Ep, 22.5 ± 0.6; endotoxin + Ep, 9.4 ± 1.4. Data represent mean ±SEM for five experiments except Ep and endotoxin + Ep groups which represent three experiments.

less prominent, but significant, increase over nontreated controls was noted in splenic CFU-E ($P < 0.001$) and Days 1 and 2 ^{59}Fe incorporation ($P < 0.01$). When compared to nontreated mice, the marrow of the endotoxin group had decreased nucleated cells, CFU-E, and Day 3 BFU-E (all three P values < 0.001) (Table I, Fig. 2). Marrow ^{59}Fe incorporation was decreased from control levels on each day, but the greatest decrease was on Day 3 ($P < 0.05$).

In contrast, the spleens of Ep-treated mice, compared to those receiving no treatment, had a large increase in CFU-E and Days 1 and 2 ^{59}Fe incorporation with a relatively smaller, but significant increase in total nucleated cells ($P < 0.05$), Day 3 BFU-E ($P < 0.01$), and Days 3 ($P < 0.01$) 4, and 5 ($P < 0.05$) ^{59}Fe incorporation into heme (Table I, Fig. 1). In the marrow of Ep-treated mice, CFU-E ($P < 0.001$) and Days 1 and 2 ^{59}Fe incorporation ($P < 0.02$) were much greater than those of nontreated controls, but marrow Day 3 BFU-E and Days 3–7 ^{59}Fe incorporation were similar to controls (Table I, Fig. 2) while the number of total nucleated cells was

slightly increased. Compared to Ep-treated mice, the spleens of mice given endotoxin had less CFU-E ($P < 0.001$) and markedly less Day 1 ^{59}Fe incorporation, yet they had very markedly increased Day 3 BFU-E and Days 3–5 ^{59}Fe incorporation.

The spleens of mice given endotoxin + Ep had nucleated cell counts that were not significantly different from those given Ep, but their CFU-E and BFU-E ($P < 0.001$) and Days 2–4 ^{59}Fe incorporations ($P < 0.01$) were greatly increased over those mice receiving Ep alone (Table I and Fig. 1). The splenic incorporation of ^{59}Fe on Days 5–7 was similar in both groups (Fig. 1). Compared to the endotoxin group, the mice treated with endotoxin + Ep had much more splenic CFU-E and Days 1 and 2 ^{59}Fe incorporation, with a relatively smaller but significant increase in nucleated cells ($P < 0.001$), but by 4 and 5 days of cell incubation the ^{59}Fe incorporation was significantly decreased ($P < 0.05$) (Fig. 1). Compared to Ep-treated mice, the marrow of mice treated with endotoxin + Ep had markedly decreased nucleated cells and CFU-E, and decreased Days 1 and 2 ^{59}Fe incorporation ($P < 0.01$). Marrow ^{59}Fe incorporation on Days 3–7 was similar in the endotoxin + Ep group and the group receiving Ep alone (Fig. 2). The marrow of mice treated with endotoxin + Ep, when compared to endotoxin-treated mice, had nucleated cell counts that were similar, but the CFU-E and Day 3 BFU-E were increased ($P < 0.001$) and Days 1–4 ^{59}Fe incorporation also was increased ($P < 0.05$) (Table I, Fig. 2).

Discussion. These studies used plethoric mice, which have decreased numbers of CFU-E (1), and concentrations of limulus-absorbed Ep *in vitro* which were suboptimal for Day 8 BFU-E development, but which allowed Day 3 BFU-E development. Under these conditions, the predominant erythroid progenitor cell assayed in mouse spleen and marrow was the Day 3 BFU-E. These BFU-E, however, are a heterogeneous population whose progeny first appear on Day 3 of culture and last through Day 5 (4). Such heterogeneity results in the continual appearance and subsequent senescence of the bursts, but these dynamic events are not assessed by the scoring of bursts which is performed at a fixed time. A parallel relation between serial CFU-E numbers and 4-hr ^{59}Fe incorporation (3), and between Day 8 BFU-E and 24-hr ^{59}Fe incorporation (17), previously has been demonstrated; and our data from Table I, when graphed per organ, show a linear relationship between Day 3 BFU-E and Day 3 ^{59}Fe incorporation for both marrow and spleen. In our studies the numbers of CFU-E and Day 3 BFU-E correlated best with Day 1 and Day 3 ^{59}Fe incorporation into heme. We used the latter method for measuring kinetic changes in erythropoiesis *in vitro* because we believe it better reflects the overall heterogeneity of the erythroid precursor cells and it allows evaluation of very late progenitors such as proerythroblasts, whose proliferative capacity is not sufficient to form colonies, but whose heme synthetic capacity is quite prominent. As we tried to assess total erythropoiesis, and as changes in the number of CFU-E and Day 3 BFU-E bore no constant relation to nucleated cell counts, all data were expressed per spleen or hind limb.

When endotoxin was administered to plethoric mice a marked increase in Day 3 BFU-E and smaller increases in CFU-E and Day 1 ^{59}Fe incorporation were observed in the spleens. At the same time the marrow had a marked decline in the numbers of CFU-E and Day 3 BFU-E. These results are consistent with a shift of these cells from the marrow to the spleen as proposed for pluripotent colony-forming cells (CFU-S) (18). However, it is also possible that endotoxin increases the replication of Day 3 BFU-E in the spleen since it has been shown to increase the number of splenic CFU-S in cell cycle (19). In contrast to the effect of endotoxin, Ep produced a marked increase in CFU-E in both the marrow and spleens of these animals with only a very slight increase in splenic Day 3 BFU-E, and it had no significant effect on the number of marrow Day 3 BFU-E.

When endotoxin was administered with Ep and the effect was compared to the effect of Ep alone it is clear that endotoxin enhanced the magnitude of the Ep effect on the spleen, producing a further increase in the number of splenic CFU-E and an even greater increase in Day 3 BFU-E. Likewise, endotoxin greatly reduced the magnitude of the Ep ef-

)n the marrow producing a marked de- in the number of CFU-E.

contrast, the effect of Ep and endotoxin .e splenic cells compared to the effect of toxin alone, was to increase the number FU-E and decrease the number of pre- ır cells that gave rise to heme synthesis ays 4 and 5. This decrease in less differ- ted precursor cells was clearly revealed .e cumulative ^{59}Fe incorporation studies eas it was not evident in the ^{59}Fe incor- ion and count of BFU-E on Day 3. out the addition of Ep, the large number lenic Day 3 BFU-E induced by endo- showed little development into CFU-E,)t what might be accounted for by small ual endogenous levels of Ep in plethoric . In the marrow, CFU-E, Day 3 BFU-E Days 1–4 ^{59}Fe incorporation were in- ed when Ep was given with endotoxin, he shift to more differentiated progeni- vas less than that seen in the spleen and isproportionate decline of Days 4 and 5 incorporation was evident.

ese experiments indicate that endotoxin ly enhances the number of Day 3 BFU- the spleens of plethoric mice, but it has effect on erythropoietic differentiation.)n the other hand, produces differentia- on erythroid progenitor cells and leads ı increased number of splenic CFU-E npanied by a decline in the number of iroid precursor cells that produce hemo- n-synthesizing erythroblasts by Days 4 ; *in vitro*. These results extend the obser- ns of Reissmann *et al.* (11) who postu- that the primary effect of endotoxin .t be to increase the numbers of splenic iroid precursor cells that preceded the -E development. The only previous re- of Ep causing an increase in CFU-E an accompanying decrease in BFU-E is of Iscove (20) who showed such a rela- between CFU-E and Day 10 BFU-E in ow of bled mice when the marrow was iined 4 days after bleeding. Our experi- s do not exclude an effect of Ep on the eplication of CFU-E, but they do sug- that the latter can be generated from oping BFU-E.

mmary. When the marrow and spleen of plethoric mice are cultured with 1.0 unit/ml of limulus-lysate-absorbed Ep, the predominant erythroid progenitor cell assayed is the Day 3 BFU-E. Erythropoiesis by these cells can be studied by enumeration of bursts at 3 days or by 24-hr ^{59}Fe incorporation into heme. Endotoxin markedly increased the number of Day 3 BFU-E in the spleens of these mice and decreased the number in the marrow, but it had little effect on erythropoietic maturation. In contrast, Ep produced differentiation of the erythroid progenitor cells resulting in increased splenic CFU-E and a decline in the more primitive erythroid precursor cells.

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ct of Vitamin E on Lipid Composition of Submandibular and Lacrimal Glands in Rats[1] (40665)

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generally believed that vitamin E is a antioxidant which prevents the lipid lation of cellular and subcellular mem- (1). In vitamin E-deficient animals, a decrease in the content of polyun- ed fatty acids (PUFA) in various tis- id subcellular organelles (2–5). Al- changes in cholesterol and total lipid of plasma and liver in rats have been d (6–10), to our knowledge, there is no ation in the literature on the effects of ve intake of vitamin E on tissue fatty mposition.

nandibular salivary glands (SMSG) raorbital lacrimal glands are both ex- secretory glands and an active lipid olism has been reported in SMSG (11– e several other tissues, the SMSG (17, d lacrimal glands (19) also undergo luced modifications in their fatty acid s. We have previously shown that in al fatty acid (EFA) deficiency the fatty mposition of various lipids in these nds is altered (18, 19). In the SMSG A-deficient rats, modification of lipid sition was associated with a reduction rate of pilocarpine-stimulated whole 18).

effects of vitamin E on the lipid com- n of SMSG and lacrimal glands have viously been investigated. Since mod- ns in the fatty acid patterns of tissue especially structural lipids, may be as- d with functional changes, the present gation was conducted to study if dif- levels of vitamin E will alter the fatty mposition of SMSG and lacrimal

rials and methods. a. Animal study. groups of 15-days pregnant, Sprague–Dawley rats[3] were fed purified diets containing three different levels of vitamin E, 0, 250, or 2500 IU vitamin E (DL-α-tocopherol acetate) per kilogram of diet. The basal diet[4] was similar to M.I.T. No. 200 (20) but without any vitamin E added. It had the following percentage composition: lactalbumin, 20.0; sucrose, 67.0; cellulose, 6.0; salt mixture 3.0; tocopherol-stripped lard, 3.0; vitamin mixture (without vitamin E), 1.0. The fatty acid composition of tocopherol-stripped lard was determined by analyzing duplicate samples using gas chromatographic methods. It contained in percent: $C_{14:0}$, 5.2; $C_{16:0}$, 21.2; $C_{16:1}$, 5.9; $C_{18:0}$, 20.0; $C_{18:1}$, 37.4; $C_{18:2}$, 9.3; and $C_{20:0}$, 1.0.

One day after birth, the pups in each group were randomized to form foster litters, each containing 8 pups. This was done in order to minimize genetic variability between the three groups and to eliminate any possible effects of litter size on lipid composition and metabolism of the tissues. Feeding of the same diets to foster mothers was continued until the pups were weaned at 19 days of age. After weaning, young rats (20/group, 10 male and 10 female) were housed in separate, wire-bottom cages and were fed *ad libitum* the same diets which had previously been fed to their foster mothers. Rats were weighed twice a week and the food intake was occasionally measured.

Rats were killed by decapitation 4 weeks after weaning, blood samples were collected in heparinized centrifuge tubes and plasma was obtained by centrifugation. SMSG and exorbital lacrimal glands were dissected out and weighed. Tissues from 6 rats in each group were used for lipid analyses; the remaining rats were used in another study.

b. Extraction of lipids. All solvents were

study was supported by Research and Grants e of LSU School of Dentistry.

or to whom all correspondence should be ad-

[3] Holtzman Co., Madison, Wis.

[4] All dietary ingredients except sucrose were purchased from ICN, Cleveland, Ohio.

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reagent grade and were freshly redistilled prior to use. Tissue or plasma samples were extracted for lipids with chloroform–methanol (2:1, by vol), using Folch's method (21). Washed lipid extracts were made to a volume (10 ml) and stored under nitrogen at −20°.

c. Analytical methods. Total lipids were determined in 0.2-ml aliquots of the lipid extract using Amenta's method (22) while phospholipids were determined in another aliquot of the lipid extract, using Bartlett's procedure (23). α-Tocopherol concentrations were determined using Bieri's method (24). For all these analytical procedures, duplicate samples of the total lipid extract were used.

Neutral lipids and phospholipids were separated by silicic acid chromatography (25). Aliquots of total lipid extract, neutral lipids and phospholipids were saponified under nitrogen by heating at 100° for 5 min with 0.5 *N* methanolic-NaOH and the methyl esters of the fatty acid were prepared using 14% BF_3-methanol (26). The methyl esters of fatty acids were extracted with hexane and the fatty acid composition was determined using gas–liquid chromatography as previously described (18).

All data were statistically evaluated using analysis of variance and Newman–Keuls' test (27). Since there was no difference in the lipid and fatty acid composition of the glands between male and female rats, the data from six rats in each group were combined.

Results. Food intake determinations made on two separate occasions during the study showed no significant differences in the three groups. Body weights and gland weights of rats fed different levels of vitamin E are shown in Table I. Final body weights of rats fed 250 IU of vitamin E/kg of diet were slightly higher as compared to the other two groups. This difference in body weight was not, however, statistically significant. The absolute weights of SMSG and lacrimal glands were also slightly higher in the rats fed 250 IU vitamin E/kg of diet. However, when expressed in terms of body weight, there was no difference in either SMSG or lacrimal gland weights in any of the three groups.

The data on total lipids and total phospholipids of the lacrimal gland and SMSG are shown in Table II. As compared to the lacrimal gland, the concentrations of total lipids in SMSG were generally higher. There was no significant difference in total lipids of either type of glands whether 0, 250, or 2500 IU/kg of diet of vitamin E were fed. Total lipid phosphorus content was the highest in SMSG and lacrimal glands of rats fed 0% dietary vitamin E as compared with the other two groups.

Table III shows the fatty acid composition of total lipids of the lacrimal glands and SMSG as affected by different dietary vi-

TABLE I. EFFECT OF DIFFERENT LEVELS OF VITAMIN E ON BODY AND TISSUE WEIGHTS

Group	Dietary vitamin E (IU/kg diet)	I. B. Wt.[a] (g)	Final B. Wt. (g)	SMSG		Lacrimal glands	
				mg	mg/100 g B. Wt.	mg	mg/100 g B. Wt.
I	0	48.4 ± 0.1[b]	166.1 ± 10.8	202.0 ± 6.8	115.5 ± 3.5	95.6 ± 3.8	54.9 ± 2.3
II	250	52.7 ± 0.8	191.1 ± 7.2	219.0 ± 8.5	114.9 ± 2.3	99.1 ± 3.4	52.4 ± 1.6
III	2500	51.8 ± 1.1	180.4 ± 5.6	203.4 ± 7.3	112.2 ± 2.7	94.3 ± 2.3	52.7 ± 1.5

[a] Body weight at the time of weaning.
[b] Values are means ± SEM of 19–20 rats/group.

TABLE II. EFFECT OF FEEDING DIFFERENT LEVELS OF VITAMIN E ON TOTAL LIPIDS AND LIPID PHOSPHORUS IN SMSG AND LACRIMAL GLANDS OF RATS

	Lacrimal gland			SMSG		
	dietary vitamin E (IU/kg diet)			dietary vitamin E (IU/kg diet)		
	0	250	2500	0	250	2500
Total lipids (%)	2.96 ± 1.18[a]	2.22 ± 0.42	2.32 ± 0.66	3.78 ± 0.51	3.39 ± 0.42	3.63 ± 0.29
Total lipid P (μmole/g)	24.8 ± 1.79	21.6* ± 1.54	20.8* ± 2.11	28.2 ± 3.77	20.8* ± 2.82	20.7* ± 1.69

[a] Values are mean ± SD of six rats/group. All values are expressed on the basis of fresh weight of the tissue.
* Significantly different as compared with 0 vitamin E group ($P < 0.01$).

ABLE III. EFFECT OF DIFFERENT LEVELS OF VITAMIN E ON THE FATTY ACID COMPOSITION OF TOTAL LIPIDS IN SMSG AND LACRIMAL GLANDS OF RATS

	Lacrimal gland			SMSG		
	dietary vitamin E (IU/kg diet)			dietary vitamin E (IU/kg diet)		
Fatty acid	0	250	2500	0	250	2500
14:0	1.4 ±0.22*	1.4 ±0.20	1.2 ±0.10	0.8 ±0.20	0.8 ±0.17	0.7 ±0.15
Iso 16:0**	1.3 ±0.17	1.4 ±0.17	1.4 ±0.17	1.7 ±0.34	1.7 ±0.15	1.6 ±0.15
16:0	24.4 ±1.49	25.8 ±0.54	24.7 ±1.59	19.5 ±0.32	19.5 ±0.56	20.0 ±0.47
16:1	14.3 ±3.09	9.6 ±3.31	11.4 ±3.87	4.4 ±0.56	4.0 ±0.64	4.3 ±0.71
18:0	9.1 ±1.71	9.65 ±0.34	10.2 ±0.51	12.9 ±0.59	12.8 ±0.44	12.9 ±0.64
18:1	23.8 ±3.67	21.2 ±2.89	22.6 ±1.15	23.6 ±1.52	22.5 ±0.78	22.9 ±1.20
18:2	6.1 ±0.34	7.4 ±0.76	7.3 ±1.30	6.3^{a} ±0.61	6.7 ±0.27	7.2^{a} ±0.54
20:1	1.5 ±0.34	1.48 ±0.59	1.8 ±0.47	1.3 ±0.17	1.3 ±0.17	1.2 ±0.17
20:2	1.0 ±0.37	1.40 ±0.66	1.1 ±0.27	0.7 ±0.10	0.8 ±0.17	0.8 ±0.27
20:3 ω9	4.42^{a} ±0.98	4.65^{b} ±0.71	$3.35^{a,b}$ ±0.59	6.1^{c} ±0.61	5.3^{a} ±0.81	$4.1^{a,c}$ ±1.15
20:3 ω6	$1.98^{a,b}$ ±0.39	2.65^{a} ±0.47	2.87^{b} ±0.61	$3.9^{c,d}$ ±0.29	4.6^{d} ±0.47	5.0^{c} ±0.32
20:4	9.2^{a} ±0.44	12.2^{a} ±1.69	10.8 ±1.94	15.9 ±0.73	17.0 ±1.67	17.2 ±1.86
20:3 ω9 / 20:3 ω6	2.22^{c} ±0.29	1.79^{b} ±0.34	$1.17^{b,c}$ ±0.39	$1.57^{a,c}$ ±0.22	1.17^{a} ±0.29	$0.82^{a,c}$ ±0.27

* Values are mean (% by weight) ± SD of six rats/group. Values sharing a common superscript letter are nificantly different using analysis of variance, a and b indicating $P < 0.05$, c and d, $P < 0.01$.
** Tentative identification.

min E levels. The level of $C_{20:3\ \omega6}$ in the total ›ids increased with an increase in dietary tamin E. However, the levels of $C_{20:3\ \omega9}$ anged in the opposite direction, i.e., with ı increase in dietary vitamin E, the levels of is fatty acid decreased significantly. As a sult of these changes in the fatty acid com-›sition, the $C_{20:3\ \omega9}$ to $C_{20:3\ \omega6}$ ratio decreased the SMSG as well as lacrimal gland total ›ids as the level of vitamin E was increased ›m 0 to 2500 IU/kg of diet. As compared the SMSG, the lacrimal gland lipids had 'o- to threefold higher proportions of $C_{16:1}$. Pooled samples of neutral lipids and phos-ıolipids from each of the three groups were so analyzed for their fatty acid composition. milar changes were observed in both ASG and lacrimal glands as were found in eir total fatty acid composition, i.e., the tios of $C_{20:3\ \omega9}$ to $C_{20:3\ \omega6}$ fatty acids were duced as the level of vitamin E in the diet as increased (data not shown).

TABLE IV. THE CONCENTRATIONS OF α-TOCOPHEROL IN PLASMA, SMSG, AND LACRIMAL GLANDS OF RATS FED DIFFERENT LEVELS OF VITAMIN E[a]

Group	Dietary vitamin E (IU/kg diet)	Plasma (mg/100 ml)	SMSG[b] (μg/g)	Lacrimal glands[b] (μg/g)
I	0	0.14^{a} ±0.05	1.3	2.7
II	250	1.02^{a} ±0.15	33.1	28.2
III	2500	3.65^{a} ±0.42	72.4	74.3

[a] Values are mean ± SD of six rats/group. Values sharing a common superscript letter are significantly different, $P < 0.01$.
[b] Pooled sample from six rats. Wet weight of the tissue.

The concentrations of α-tocopherol in plasma, SMSG, and lacrimal glands of rats from this study are shown in Table IV. The plasma levels of α-tocopherol were the lowest

in the group fed vitamin E-deficient diet. The concentrations of α-tocopherol in plasma increased from 0.14 to 1.02 to 3.65 mg/100 ml as the diet contained increasing amounts of vitamin E. Similarly, the concentrations of α-tocopherol in the lacrimal gland and SMSG increased from 27- to 56-fold with an increase in dietary vitamin E levels.

Discussion. The lower level of vitamin E (250 IU/kg) used in our study is about eightfold the requirement of vitamin E for rats (28). However, comparable levels of vitamin E, 200–500 IU/kg, have been used by other investigators in their control diets to study the effects of vitamin E on tissue lipid composition (29) and on liver microsomal fatty acid desaturases (30) in rats.

The excessive intake of vitamin E has been reported to result in decreased thymus weight and increased adrenal weight in rats (31), and adrenal degeneration and growth inhibition in chickens (32). Reduced body weights, increased relative heart, and spleen weights have also been described as some of the adverse effects of excess vitamin E in rats (33). The lack of significant changes in body weights and SMSG and lacrimal gland weights in groups fed excess vitamin E in our study may be due to comparatively shorter experimental duration and differences in vitamin E levels. Whereas, we fed the different diets for a total of about 7 weeks (1 week during pregnancy, 19 days of lactation, and 4 weeks postweaning), in some of the studies which have reported changes in body weights and organ weights of animals, much longer experimental periods of up to 16 months and higher levels of vitamin E, 10,000 and 25,000 IU/kg of diet have been used (10).

Dietary level of vitamin E did not have an effect on the total lipid content of the SMSG and lacrimal gland. This differs with the results obtained on liver (7) where an increase in dietary level of vitamin E decreased the total lipids.

Increasing the dietary levels of vitamin E resulted in higher concentrations of vitamin E in plasma, SMSG, and lacrimal glands. Increases were proportional to the dietary levels of vitamin E. Similar findings have been reported in other tissues such as liver (10) and red blood cells (34). It would be interesting to study how changes in the level of vitamin E in SMSG and lacrimal glands influence the excretion of this vitamin in saliva or lacrimal fluid.

Although we did not use criteria such as susceptibility of red blood cells to hemolysis *in vitro* to assess vitamin E deficiency, rats fed 0 IU vitamin E had very low levels of vitamin E in plasma (0.14 mg/100 ml) and in the SMSG and lacrimal glands, thus indicating that these rats were indeed deficient in vitamin E since plasma levels less than 0.5 mg/100 ml are generally associated with vitamin E deficiency and those above 1 mg/100 ml are known to reflect adequate vitamin E nutrition (35).

In vitamin E-deficient animals, the levels of PUFA in various tissues and subcellular organelles is decreased (2–5). This may result in an increased tendency for lipid peroxidation of the membranes, resulting in cellular damage. What effect does an excess of vitamin E have on the profiles of PUFA has not, however, been investigated. Our studies indicate that, at least in the SMSG and lacrimal glands of rats, excess vitamin E can modify PUFA composition. There was an increase in the levels of $C_{20:3\ \omega6}$ and a decrease in $C_{20:3\ \omega9}$ levels as the dietary vitamin E was increased. This resulted in a reduction in the ratio of $C_{20:3\ \omega9}$ to $C_{20:3\ \omega6}$. Since our basal diet was marginally deficient in EFA which provided 0.7% of the total calories rather than a minimum of 1% for the rat (36), it might have influenced the fatty acid composition of the lipids such as relatively high 20:3 ω9 levels which were observed in all the three groups.

One mode of action of vitamin E in terms of its ability to protect membranes from peroxidative damage is that it can form a complex with arachidonic acid residues of membrane phospholipids. It has been proposed (37) that the two methyl groups at C_4' and C_8' of the phytyl side chains of vitamin E can fit in the pockets created by the cis double bonds at C-5 and C-11 of arachidonic acid, $C_{20:4\ \omega6}$. It is possible that other PUFA with similar structural features may also interact with vitamin E. Our data give some indirect evidence that this type of interaction is likely to take place between vitamin E and $C_{20:3\ \omega6}$ since the levels of this fatty acid increased in both SMSG and lacrimal glands as the levels of vitamin E in the diet and consequently in

inds were increased. Since $C_{20:3\ \omega6}$ and
are structurally similar in terms of
cis double bonds at carbons 8, 11, and
the levels of both of these fatty acids
glands increased with an increase in
t-induced tissue levels of vitamin E, it
er tempting to speculate that the struc-
equirements for complex formation be-
the two methyl groups of vitamin E at
d C_8' of the phytyl side chain and the
ible bonds of the unsaturated fatty acid
t at C-5 and C-11 as proposed by Lucy
it rather at C-8 and C-14 of the PUFA.
ther mechanism whereby the different
of dietary vitamin E could have af-
the lipid composition of the tissues
ed in our present investigation is by
icing the circulating thyroid hormone
Although we did not measure the
a thyroid hormone levels in these rats,
been shown that excessive intake of
n E suppresses thyroid activity in
n (38) and reduces the serum thyroid
ne levels in man (39). A number of
have indicated that thyroid hormones
dly affect lipid metabolism in man and
eral species of animals. Thyroxine ad-
ration to normal rats increases the he-
oncentrations of all fatty acids, espe-
those in phospholipid and free fatty
ractions (40). Therefore, it is possible
he lower phospholipid concentrations
ed by us in the SMSG and lacrimal
of rats fed vitamin E-supplemented
nay be due to lower thyroid hormone

activities of microsomal Δ9 and Δ6
rases are modified in different ways by
etary components. Whereas, the 9-de-
se activity is increased by a high car-
rate diet, a high protein diet increases
desaturation (41–44). *In vitro* stimula-
f microsomal Δ9 desaturation activity
en observed in thyroxine-treated rats
5). The Δ6 desaturase activity seems to
ibited by thyroxine (46). In case the
ies of these two desaturases in the
and the lacrimal glands are also sim-
effected by thyroid hormones it is quite
le that this may have resulted in de-
d synthesis of 20:3 ω9 and increased
sis of 20:3 ω6, thus resulting in lower
rtions of 20:3 ω9 to 20:3 ω6, with an increase in vitamin E levels. There is also some evidence that the activity of hepatic microsomal Δ9 desaturase is decreased in rats fed for 10 weeks diets deficient in vitamin E or containing supplemental levels of vitamin E (30).

Summary. The effects of different levels of vitamin E on SMSG and lacrimal gland lipids were studied in rats. Feeding of purified diets containing 0, 250, or 2500 IU vitamin E/kg of diet was initiated during gestation and continued throughout lactation and then to the pups until 1 month after weaning. SMSG and lacrimal glands were extracted for lipids and the effects of vitamin E on lipid and fatty acid composition were examined. Total lipid content was not affected by different levels of vitamin E. Total lipid phosphorus was, however, decreased with an increase in dietary vitamin E. Vitamin E content of the SMSG and lacrimal glands was increased by severalfold depending upon the level of dietary vitamin E. The fatty acid composition of total lipids, neutral lipids, and phospholipids showed that the levels of $C_{20:3\ \omega9}$ were reduced and those of $C_{20:3\ \omega6}$ were increased as the dietary levels of vitamin E were increased. This resulted in a decrease in the ratio of $C_{20:3\ \omega9}$ to $C_{20:3\ \omega6}$.

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ıine in Fetuses after Maternal Injection: Increasing Concentration with Advancing Gestational Age (40666)

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opioids are used clinically and abu-
during human pregnancy. It is impor-
know the drug concentrations in fe-
f varying gestational age after admin-
n of the drug to the mother.
ral previous studies have shown the
t of opiates crossing the placenta in
ring late gestation (19 and 21 days).
lerable quantities of dihydromorphine
he placenta at 19 and 21 days of ges-
(1, 2). Sixteen hours after injection,
ain still contains measurable amounts
ydromorphine while maternal brain
s only trace amounts (1). When di-
norphine is injected into "tolerant"
the 21st day of gestation the peak
tration found in the fetuses is earlier
nontolerant fetuses (3).
ntly, Davis and Fenimore (4) have
that very little methadone crosses the
ta during early gestation in monkeys,
uivalent concentrations are found in
al and fetal tissues during late gesta-

present study was undertaken to de-
e the amount of morphine in rat fetuses
ious days of gestation after subcuta-
injection to the dam, the time intervals
njection that morphine can be meas-
the fetuses, and to compare morphine
in 15-day fetuses of dams which were
nd those chronically exposed to mor-

erials and methods. Pregnant Wistar
veighing approximately 200 g) were
d from Charles River Laboratories on
day after conception. The rats were
singly in wire-bottomed cages in a 12-
t–dark cycle and a temperature- and
ity-controlled environment. At 10:00
gestation Days 11, 13, 15, 17, and 19,
ts for each day were injected s.c. with
f physiological saline containing 180
f n-[1-^{3}H]morphine 28 Ci/mmole
sham) with an appropriate amount of nonradioactive morphine sulfate (Mallinckrodt) to result in a final morphine dose of 4.8 mg/kg (expressed as free base) adjusted for the weight of each animal. This dose has been shown to be an analgetic dose of morphine in the rat (5) and affects fetal activity *in utero* (6). The purity of the labeled morphine had been checked previously by thin-layer chromatography (absolute ethanol:acetic acid: water, 6:3:1) which showed that 93% of the radioactivity was associated with morphine.

At 30 min, 1, 2, 6, and 12 hr after injection the rats were anesthetized with ether and the fetuses were delivered by Caesarean section, frozen in liquid nitrogen, and stored in liquid nitorgen until the tissues could be analyzed. Eight individual whole fetuses were analyzed at each time on Day 11 of gestation. Five individual fetuses from each time on Days 13, 15, 17, and 19 were divided into head and trunk pieces which were assayed separately. Five placentas for each time and three pieces of maternal liver (always taken from the same lobe) and part of the maternal brain, divided into lower brainstem and diencephalon/caudate pieces were also assayed. One additional pregnant rat from each day was not injected, and the same tissues were analyzed to obtain blank values.

One group of five pregnant rats was chronically injected with morphine prior to the experiment. Beginning on Day 7 of gestation these rats were injected with 1 ml of nonradioactive morphine in normal saline s.c. in the shoulder region, every 12 hr (10:00 AM and 10:00 PM). The first two injections were 5 mg/kg (expressed as free base) and all subsequent injections were 10 mg/kg until Day 15. On Day 15 of gestation these rats were injected with tritiated morphine at 10:00 AM and processed as described above.

The frozen tissues were weighed and homogenized in 1 vol of absolute ethanol in glass homogenizers. The resulting homogenate was spun in a clinical centrifuge and 50

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μl of the supernatant was added to 10 μl of cold morphine carrier (1.5 mg/ml). Fifty microliters of this solution was spotted onto Gelman silica gel ITLC sheets with fluorescent indicator, and developed with absolute ethanol:acetic acid:water (6:3:1). The R_f for morphine in this sysgem is 27 and has been reported to be different from the Rfs of the major metabolites (7). The plates were dried and the morphine spots visualized under a fluorescent lamp. The spots were cut out and placed in scintillation vials with 5 ml of scintillation cocktail and counted in a Beckman Model LS-230 liquid scintillation counter. Recovery of labeled morphine from the homogenates was 90%.

Various dilutions of the injection solution were made with absolute ethanol or water and taken through the analytical procedure. Known amounts were taken from the aqueous dilutions and added to liver homogenates and analyzed. In all cases a linear relationship was observed when net dpm values were plotted against the volume of the labeled injection solution. Taking the recovery of the morphine and various dilutions during the assay procedure into consideration, it was calculated that 1 ng of labeled morphine corresponded to 178.15 ± 4.54 dpm (SEM). The results (ng/wet wt of tissue) refer to the total amount of free morphine only.

The data was analyzed by an analysis of variance for each tissue on individual days. The concentration of morphine in the head and body were found to be the same by a Student's t test and were pooled and compared by an analysis of variance for each time after injection.

Results. There was no difference in the amount of morphine in the placenta (per gram of tissue) at the same time after injection, regardless of the day of gestation and these values were pooled (Table I). The peak concentration of morphine in the placenta was at 1 hr after injection. This dropped sharply by 6 hr and was about 100 ng/g by 12 hr after injection.

The concentration of morphine in Day 11 fetuses was highest (65 ng/g) at 1 and 2 hr, and dropped to negligible values by 12 hr after injection (Table I). The peak morphine concentration in the fetuses was progressively higher on Days 13, 15, 17, and 19 ($P < 0.001$). These concentrations represented only a fraction of 1% of the dose administered. The highest morphine concentration in the fetuses was observed at Day 17, 1 hr after drug administration to the mother. At this time, morphine concentration in the fetuses represented 65% of that in the placenta. The peak concentration on Day 19 represented only 72% of the peak concentration at Day 17. On all of the days measured, the concentrations in the fetuses dropped markedly by 6 hr and were quite low by 12 hr after injection although morphine was still measurable.

The peak concentrations in maternal tissues were at 1 hr (Table I). These include maternal liver, brainstem, and caudate-diencephalon pieces. The peak concentration

TABLE I. MORPHINE CONCENTRATION (ng/g wet wt) IN FETAL AND MATERNAL TISSUES AT VARIOUS TIMES AFTER SUBCUTANEOUS INJECTIONS OF MORPHINE TO THE MOTHER

	Hours after injection				
	½	1	2	6	12
Fetal tissue					
Day of Gestation					
11	9.9 ± 1.9[a]	63.5 ± 8.5	54.1 ± 9.8	43.4 ± 8.5	2.2 ± 1.5
13	192.5 ± 29.1	179.8 ± 21.3	168.7 ± 19.6	15.0 ± 5.0	11.0 ± 4.5
15	243.9 ± 17.1	405.9 ± 10.3	396.0 ± 13.0	85.4 ± 5.6	64.7 ± 3.6
17	176.2 ± 9.1	714.2 ± 31.8	461.2 ± 15.4	76.7 ± 3.7	54.3 ± 8.1
19	356.4 ± 2.0	519.6 ± 17.9	367.5 ± 21.4	183.7 ± 9.8	76.5 ± 6.4
Placenta	746.4 ± 41.4	1092.2 ± 100.9	721.7 ± 73.3	182.4 ± 17.5	100.5 ± 6.4
Maternal tissues					
Liver	904.3 ± 55.0	1442.7 ± 171.0	713.0 ± 91.6	626.9 ± 53.1	329.2 ± 51.9
Brainstem	144.6 ± 12.1	262.3 ± 29.9	83.2 ± 16.6	12.5 ± 3.8	37.3 ± 3.9
Diencephalon	87.6 ± 7.6	170.0 ± 47.2	72.3 ± 6.7	14.7 ± 2.9	5.6 ± 3.0

[a] Variation is expressed as SEM.

MORPHINE CONCENTRATION (ng/g wet wt) IN FETUSES AND PLACENTAS ON DAY 15 OF GESTATION AFTER CHRONIC EXPOSURE TO MORPHINE

	½	1	2	6	12
	561.0 ± 31.1[a]	456.8 ± 35.6	319.9 ± 19.3	72.1 ± 6.7	47.2 ± 5.7
age of acute	230	113	80	84	73
	1372.4 ± 53.1	1167.6 ± 47.1	583.0 ± 27.8	166.7 ± 20.0	63.8 ± 3.8
age of acute	181	107	81	91	63

[a] is expressed as SEM.

as 5.5 times higher than the peak concentration. Twelve hours after he maternal brain tissues contained amounts of morphine while the entration was still higher than any er tissues analyzed.

ally injected animals measured on gestation showed a peak concen- ½ hr after injection (Table II). The ion was 230% higher than in naive hr after injection. By 1 hr after he morphine in chronically injected as 113% that of naive animals. At 12 hr the concentration in chroni- sed fetuses and placentas was less ive fetuses.

on. The major finding of this paper, hine passes the placenta with de- ifficulty as gestation progresses, is with the morphological develop- ie placenta. In the rat the placenta orial. The villi are bathed in mater- . This arrangement provides two ompartments, i.e., the fetus and the ith a barrier in between. The mor- l barrier separating maternal from l consists of (i) synctiotrophoblast, phoblast, (iii) basal lamina of the blast cells, (iv) mesenchyme, (v) ina, and (vi) endothelium of fetal . Early in pregnancy this barrier is thick and becomes much thinner as progresses. The cytotrophoblast ppears altogether leaving the sync- last and basal lamina (8). In addi- ig pregnancy there is a progressive n the diameter of the terminal villi responding increase in their num- esults in a larger functional surface naternal exchange (9).

w vital materials cross the placenta diffusion. However, drugs are cross mainly by diffusion (10). placental membrane is lipoprotein er, the rate of diffusion of a drug is dependent on its lipid/water partition ratio. Thus drugs with a high lipid solubility are transferred most rapidly. In this respect the placental barrier resembles the blood brain barrier and the gastrointestinal barrier (11). Drugs with a molecular weight up to 600, M_r are thought to cross readily, but this is dependent on several factors, including pH (affecting dissociation), rate of administration, concentration in maternal plasma, and rate of metabolism of the drug by the mother, placenta, and fetus (11).

Several studies have shown that different opioids pass readily from maternal blood into the fetus at 21 days of gestation (1–3). As shown in the present study, this is not the case during earlier stages of gestation. There is also some difference in the times of peak concentrations of different opioids. Blane and Dobbs (2) found a peak of etorphine in the fetus at 1 hr after injection while the peak for dihydromorphine was at 2 hr after injection. Etorphine reaches its peak concentration in maternal blood and brain more rapidly than dihydromorphine. Etorphine has a higher lipid solubility than dihydromorphine (2).

Very low levels of the enzymes catalyzing glucuronidation are detectable in the livers from fetal and newborn rats (12, 13). However, morphine glucuronyl transferase activity of 3-day-old rats reaches the adult level (14). Yeh and Woods (3) found a significant amount of conjugated [^{3}H]dihydromorphine in 21-day fetal rats and speculated that this probably originated from synthesis in both the placenta and fetus. In the present study there was a statistically significant drop in free morphine in the fetus between Days 17 and 19 of gestation which may be accounted for by an increase in morphine conjugation to morphine glucuronide.

The peak concentration of morphine was at 30 min after injection in 15-day fetuses chronically exposed to morphine. In fetuses exposed acutely to morphine the peak con-

centration was achieved at 1 hr after injection. This result is consistent with that of Yeh and Woods (3) who found earlier peak concentrations of dihydromorphine (1 hr in tolerant, 2 hr in nontolerant) of 21-day fetuses. Yeh and Woods (3) measured the disappearance of [^{3}H]dihydromorphine from the injection site and found that disappearance is more rapid in tolerant than in nontolerant animals. This might be caused by either an increase in vascularity or blood flow through the area or both.

Summary. Pregnant rats were injected s.c. with [^{3}H]morphine on Days 11, 13, 15, 17, and 19 of gestation. At ½, 1, 2, 6, and 12 hr after injection the fetuses were delivered by Caesarean section and were analyzed for [^{3}H]morphine along with placentas and various maternal tissues. Very little morphine was found in the fetuses on Day 11. On each subsequent day until Day 17, the amount of morphine increased in the fetuses. On Day 19 the morphine in the fetuses was lower than on Day 17, possibly because of morphine metabolism by the placenta and fetus. Peak concentrations of morphine occurred in the fetuses at 1 to 2 hr after injection up to Day 15, while peaks occurred on Days 17 and 19 at 1 hr after injection. In addition, one group of pregnant animals was injected chronically with 10 mg/kg of morphine twice daily between Days 7 and 15 of gestation. On Day 15, these animals were injected with [^{3}H]morphine and the fetuses analyzed at the times stated above. These fetuses showed a peak concentration at ½ hr after injection which was higher than the 1- to 2-hr peak in naive fetuses on the same day. In conclusion, these results show that morphine passes through the placenta more easily as gestation progresses and that the concentration of morphine in the fetuses can be altered by prior morphine exposure.

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fects of Hypophysectomy and TSH on Thyroidal Fucokinase in Rats (40667)

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e is a marked heterogeneity in the rdrate moieties of thyroglobulin (1), role of these carbohydrates in the n of thyroglobulin as a substrate for hormone production and storage (2, urrently unclear. One sugar of the obulin molecule, L-fucose, is unique : it is a 6-deoxy sugar found at the acing termini of a number of oligosac- chains (4). Previous studies, which own that terminal sialyl (5) or fucosyl ips on glycoproteins can control met- unction, suggest that the terminal fu- sidues of thyroglobulin may in part the further utilization of this reposi- : thyroid hormones. We have begun nination of the enzymes involved in netabolism with a view to determining r these enzymes exert control of thy- lin synthesis and degradation and of thyroid hormone storage and re- The first such enzyme, fucokinase, was first studied in pig liver (7) and we have detected and isolated from thyroid (8). This enzyme converts L- o β-L-fucose 1-phosphate (7, 8), a first its ultimate incorporation into thyro- n (9). In this paper, we report the of hypophysectomy and TSH admin- n on fucokinase activity in rat thyroid

rials and methods. Rat fucokinase was by the disk assay used for canine fucokinase in which conversion of ose to [^{14}C]fucose-1-phosphate was d by absorption of the phosphorylated n an ion-exchange disk subsequently l by liquid scintillation techniques (8). say solution contained the following s in a total volume of 0.3 ml: 6 m*M* , 5 m*M* ATP, 8.33 m*M* KF, 3.33 m*M* ıreitol, 90 m*M* morpholinopropane : acid (MOPS) buffer, pH 6.5, and 26.7 μ*M* L-[1-^{14}C]fucose containing 0.5 μCi of radioactivity per assay volume. The enzyme solutions were assayed for 0-, 30-, 60- and 90-min periods. One unit of fucokinase activity is defined as the amount which is required to catalyze the formation of 1 nmole of L-fucose-1-phosphate per 20 min at 37°.

In order to establish that concentrations of L-fucose and ATP-Mg were at appropriate levels, double-reciprocal plots (10) were constructed for the rat enzyme. K_m values of 4.8 × 10^{-3} *M* and 2.3 × 10^{-6} *M* were obtained for ATP and L-fucose, respectively, under the conditions of the assay with crude extracts (18,000 *g* supernatant solution) of enzyme or with enzyme passed through a Bio-Gel P-2 column (data not shown).

We report the results of experiments in which we used the following two protocols: I. For studies in which the thyroid fucokinase levels of intact, hypophysectomized, or hypophysectomized-TSH-treated rats were compared (Table I), 66 male Sprague–Dawley rats, of which 27 were intact (Sprague–Dawley Co., Madison, Wis.) and 39 had been hypophysectomized just before shipment (Hormone Assay Laboratories, Chicago, Ill.), were maintained on water and Purina chow, *ad libitum*, in an air-conditioned windowless room lighted from 0800 to 2000 hr. After maintenance for 18 days, the rats were divided into three groups, as follows: 27 intact rats ("intact") and 22 hypophysectomized rats ("hypophysectomized") were given injections (i.p.) of 0.5 ml 0.9% saline at 48 and 24 hr prior to sacrifice; the remaining 17 hypophysectomized rats ("hypophysectomized-TSH-treated") were given injections of 1.0 unit of TSH (Armour Thytropar, Armour Pharmaceutical Co., Phoenix, Arizona) in 0.9% saline at 48 and 24 hr prior to sacrifice. II. The protocol for determining the fucokinase response to TSH in intact rats is given in Table II. Rats were maintained as described above.

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TABLE I. THYROID FUCOKINASE ACTIVITY IN INTACT, HYPOPHYSECTOMIZED, AND HYPOPHYSECTOMIZED-TSH-TREATED RATS

Rat treatment[a]	Fucokinase activity[b] (units/mg DNA)		Percentage of activity in intact rats			
			Crude enzyme		P-2 enzyme	
	Crude enzyme	P-2 enzyme	%	P[c]	%	P[c]
Intact	0.37 ± 0.02	0.20 ± 0.04	100	—	100	—
Hypophysectomized	0.21 ± 0.05	0.09 ± 0.03	57	<0.025	45	<0.025
Hypophysectomized TSH treated	0.70 ± 0.28	0.18 ± 0.04	189	NS	90	NS

[a] Protocol I of materials and methods.
[b] For each treatment, this represents the mean ± SD for three groups of rats with five or more rats/group.
[c] Determined from Student's *t* test to assess difference from activity in intact rats. NS, not significant ($P > 0.05$).

TABLE II. FUCOKINASE RESPONSE TO TSH IN INTACT RATS

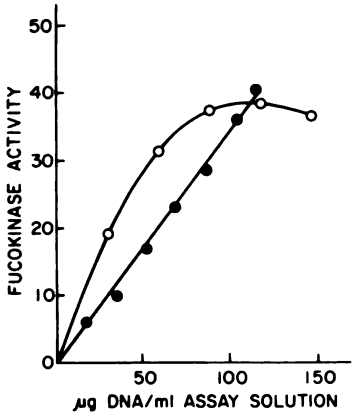
Expt No.[a]	Number of rats			Fucokinase activity[b] ratio intact TSH treated[c]
	Intact	Intact TSH treated	Sex	Intact
1	6	6	F	2.2
2	7	6	F	1.5
3	6	6	M	2.1

[a] In experiments 1 and 2, each rat of the "intact-TSH-treated" group was injected (i.p.) with 1.67 units of TSH in 0.9% saline at 25, 17, and 2 hr prior to sacrifice while each rat of the "intact" group was injected at these times with 0.9% saline only. In experiment 3, each rat of the "intact-TSH-treated" group was injected (i.p.) with 1.0 unit TSH in 0.9% saline at 48 and 24 hr prior to sacrifice while each rat of the "intact" group was injected at these times with 0.9% saline only.
[b] Thyroid fucokinase activity per animal was determined for homogenates treated with Bio-Gel P-2 (Materials and Methods). The fucokinase activity of intact rats is given in Table I.
[c] The differences between intact and TSH-treated rats were significant in all cases ($P < 0.025$).

All sacrifices were made between 0900 and 1100 hr to minimize possible effects of diurnal variation on fucokinase activity. The rats were anesthetized with sodium pentobarbital. The thyroid glands were removed from the rats while still attached to sections of the trachea and were immediately dropped into a homogenization buffer (0.02 *M* MOPS, 0.25 *M* sucrose, 0.005 *M* β-mercaptoethanol, pH 6.91). Tissues and tissue preparations were kept at a temperature of 2° unless noted otherwise. After the trachea sections had been collected from all animals, the thyroids were excised and cleaned free of fat, external blood vessels, membranes, and parathyroids on an iced watch glass under an illuminated magnifier. In order to provide sufficient enzyme activity (8) for assay, the thyroids from five or more rats of a given group were combined prior to homogenization in an ice-cooled Potter–Elvehjem homogenizer containing 0.4 ml of the homogenization buffer. Each homogenate was washed into a preweighed centrifuge tube so that the final volume was 1.0 ml (by weight). An aliquot (0.1 ml) was stored at −10° for later assay of DNA by a procedure, adapted especially for use in thyroid tissue (11), in which we used salmon sperm DNA as the standard. The remaining homogenate, 0.9 ml, was centrifuged for 15 min at 18,000 *g*, and the supernatant solution was decanted and retained. The pellet was resuspended in 0.2 ml of the homogenization buffer, and the suspension was centrifuged as before. The 18,000 *g* supernatants were combined, 0.1 ml was removed for immediate fucokinase assay, and the remainder was transferred to the top of a Bio-Gel P-2 column having a 27-ml bed volume. The original container and the transferring syringe were washed with 0.2 ml of the homogenization buffer and the wash was also transferred to the top of the gel column. The column, which had been preequilibrated with the homogenization buffer, was eluted with the same buffer, and 1-ml fractions were collected. After $A_{280\ nm}$ determination, the fractions containing protein were combined and concentrated to 0.2 ml in a Minicon B-15 Macrosolute Concentrator (Amicon Corporation, Lexington, Mass.). The retentate was transferred to a preweighed glass vial. The ultrafilter surface was washed with small aliquots of homogenization buffer, and the washes were combined with the retentate to

ı a final volume of 0.45 ml (by weight). enzyme preparation was later assayed cokinase activity. The rat thyroid fuco- : preparations were made on a stand- :d time schedule (min) such that each vas begun as follows: anesthetic injec-); trachea removal, 20; thyroid excision, ıyroid cleaning, 60; homogenization, 18,000 *g* centrifugation, 150; assay of inase in 18,000 *g* supernatant solution, Bio-Gel P-2 gel filtration, 220; Minicon concentration, 250; assay of fucokinase nicon retentate, 450.

ults. It is essential in a comparison of ıe activities between tissues from differ- ıimals that any potential effector sub- s that might modify enzyme activity be 'ed. Both positive and negative effectors okinase activity have been identified g the nucleotides and nucleotide deriv- normally present in crude tissue ex- (12). Consequently it was not surprising 8,000 *g* supernatant extracts of thyroid showed a nonlinear response when as- for fucokinase activity as a function of ıe concentration (Fig. 1). It was neces- herefore, to remove these or other ef- s from the extracts in a rapid and con- manner so that comparisons between samples could still be drawn. This was ıplished by a molecular sieving proce- vith a Bio-Gel P-2 column followed by



1. Demonstration that the removal of small les from rat thyroid extracts provides a fucokinase ıtion whose expressed activity (here as pmole of l-phosphate formed in 20 min) is a linear function ıuantity of extract used in the assay. (○) 18,000 *g* ıtant; (●) preparation treated with Bio-Gel P-2.

solution concentration with an Amicon membrane concentrator (Materials and Methods). The result of this approach is seen in Fig. 1, where a linear response was observed with P-2-treated extracts when fucokinase activity was plotted as a function of enzyme concentration.

With these procedures, hypophysectomy appreciably reduced thyroid fucokinase activity whether the enzyme was measured directly in 18,000 *g* extracts or in preparations treated with Bio-Gel P-2 (Table I). Fucokinase levels appeared to be approximately 45–55% lower after removal of the hypophysis. Treatment of hypophysectomized animals with TSH restored thyroid fucokinase activity essentially to normal (Table I). In intact animals, treatment with TSH increased thyroid fucokinase activity significantly (Table II).

Discussion. These studies provide strong evidence that rat thyroid fucokinase is under hormonal control. The 45–55% decrease of thyroid fucokinase activity in hypophysectomized rats suggests that TSH may be responsible for maintaining appropriate fucokinase levels in the thyroid. The restoration of thyroid fucokinase activity under TSH replacement therapy in hypophysectomized rats and the response of thyroid fucokinase to TSH in intact rats pinpoint TSH as the responsible hormone. Our results parallel those of Pavlovic-Hournac *et al.* (13, 14), who observed that the absolute rate of thyroglobulin synthesis, but not of general thyroid protein synthesis, decreased by 50–60% after hypophysectomy and was restored by TSH to a nearly normal rate. Thus there is a marked correlation between changes in thyroid fucokinase activity and changes in thyroglobulin synthesis. This observation supports the hypothesis that TSH can modulate thyroglobulin synthesis by regulating the level of enzymes leading to its synthesis.

Only two pathways for the biosynthesis of GDP-fucose, the immediate precursor to the formation of fucosylated glycoproteins (15), have been described for mammalian tissues: a. GDP-D-mannose → GDP-4-keto-6-deoxy-D-mannose → GDP-L-fucose (16); b. L-fucose → β-L-fucose 1-phosphate → GDP-L-fucose (7, 17). Of the enzymes that catalyze these reactions, the only one detected and studied in thyroid tissue thus far is fucokinase

(8). It is probable that fucokinase exists in thyroid tissues in all species, where it serves to convert cellular fucose to fucose 1-phosphate. This cellular fucose might originate from exogenous sources (diet, activity of intestinal flora, release from other body tissues) or from the action of a fucosidase (18) on thyroid glycoproteins such as thyroglobulin, which contains from 13 to 26 fucose residues per molecule in all of the eight species examined (19, 20). Thus one role for thyroid fucokinase would be to act as a salvage enzyme for fucose endogenous to the thyroid. A system for the *de novo* synthesis of GDP-fucose, as found in some tissues (16), has not yet been detected in thyroid tissue, but work is under way to determine whether such a system is present and whether it contains responsive enzymatic steps.

Summary. The purpose of the present investigation was to determine whether rat thyroid fucokinase is responsive to hypophysectomy and to administration of thyroid stimulating hormone (TSH). Prior to measurements of fucokinase activity in thyroid extracts, any potential low-molecular-weight effectors were removed from the extracts by a molecular sieving procedure. With this procedure, thyroid fucokinase activity in hypophysectomized rats was found to be 55% lower than in intact rats. Injections (i.p.) of TSH into the hypophysectomized rats, however, restored the activity lost upon hypophysectomy to levels that were not significantly different from those of intact animals. Also, when intact rats were given injections (i.p.) of TSH, thyroid fucokinase activity was elevated significantly above that found in intact animals given injections of carrier only.

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ɔecies Specificity in the Immunologic Reactions and Biological Functions of Transcobalamin II (40668)[1]

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the first studies of this topic it was d that antibody made against human transcobalamin (TC II), a plasma rt protein for cobalamin (Cbl), did ɔt with TC II from 4 mammals other imates (1). The TC II from the non- ; species failed to promote Cbl uptake a çells (1), an *in vitro* model used to ɔ the function of TC II (2). There has ıly one other fully reported, compre- study of the subject with results ɔ a degree conflicted with those of our ıtudy (3). The antibody for the more tudy (3) was made against rabbit TC lid react with the TC II of 14 species mals but not with the TC II of other ıtes. Isolated TC II of the rabbit pro- he uptake of Cbl by the reticulocytes ɔcies of mammals. Wright and Allen l different degrees of immunoreactiv- species of TC II with antibody from s (4).

ɔ are also conflicting data from limited of the function of TC II from a single in cells of another. The earliest true f the effect of a substance in human n the promotion of Cbl uptake by cells, HeLa cells, observed species ity (5). The uptake of monkey TC II- human HeLa cells was 1/3–1/2 that an TC II-Cbl (6). The uptake of rat Cbl by HeLa cells was 10% that of TC II-Cbl (7). Murine fibroblasts p human and murine TC II-Cbl well (8). Murine L121D leukemia ık up human TC II by processes analogous to the entry of human TC II-Cbl into human cells (9), but the investigators did not compare the degree of uptake with that of murine TC II. Isolated rat liver mitochondria took up Cbl from rat serum to a greater degree than from human serum even without accounting for the holo TC II in the sera (10). In a somewhat different approach, the fates of doubly labeled human and rabbit TC II-Cbl were similar when infused back into the rabbit (11).

Resolution of the controversy outlined above is important if models used to study the function of TC II are to cross species lines. Certain technical aspects of the past studies have not been ideal especially in the cell-uptake experiments. No attention has been paid to the effect of native Cbl bound to TC II which is abundant in the sera of some animals and thereby diluting the uptake of any apo TC II labeled *in vitro*, the only component actually measured in the past. The present study is essentially a duplication of ours of a decade ago, but with considerably improved as well as different techniques.

Materials and methods. Materials. The sera were obtained either directly from the appropriate species or purchased and stored at −20° until use. The CN [^{57}Co]Cbl, nonradioactive Cbl, rabbit antibody against pure human TC II, and cultural cells have been described previously (2, 7, 8, 12–15).

Methods. A previously published method of radioimmunoassay (RIA) for human TC II (12) was used to determine the reactivity of each TC II against antihuman TC II. The standard curve was set up as described and each whole serum assayed was adjusted in volume to provide 100 pg of TC II-Cbl. The complete panel of sera was studied in duplicate on three occasions.

Prior to the measurement of the cell uptake of each TC II-Cbl, the holo and apo TC II were measured in each serum. Apo TC II was

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measured by saturation of the serum with CN [^{57}Co]Cbl followed by separation of the TC II by 1.96 *M* $(NH_4)_2SO_4$ (13). Total TC II was measured by saturation of the TC II of each serum with CN [^{57}Co]Cbl, precipitating the TC II with $(NH_4)_2SO_4$ and measuring the Cbl by bioassay with *Euglena gracilis* (14).

All cell uptake studies were performed with TC II-Cbl totally in the holo form but with part of the Cbl attached to the TC II *in vivo* through natural Cbl metabolism and part attached *in vitro*. The complex taken up by the cells was measured by counting the CN [^{57}Co]Cbl used in the *in vitro* conversion of the mixed apo and holo TC II to all holo TC II. The calculation of uptake, however, was in terms of the *total* TC II to which the cells were exposed and the amount of *total* TC II taken up. The TC II-Cbl of each serum was isolated by precipitation with $(NH_4)_2SO_4$ and the cells were exposed to this fraction of the serum only. The human TC II-Cbl studied for comparison was prepared in the same way or was partially purified as before (2).

Uptake by RPMI lymphocytes, HeLa cells or L-929 cells was measured as described (2, 8). For each experiment 3×10^6 lymphocytes, $2.5–3.0 \times 10^6$ HeLa cells, or $\pm 3 \times 10^6$ L-929 murine fibroblasts were exposed to 500 pg of TC II-Cbl for 3 hr at 37° in M-199 medium.

Results. The amounts of holo, apo, and total TC II in each serum are shown in Table I. The group values for normal human serum were derived from two studies (13, 14) and are included for comparison only. TC II in the animal sera was identified by physicochemical means. (With the exception of canine TC II, which has been studied in detail (16), the *function* of the TC II of other species has not been shown to be the equivalent of human TC II but is assumed to be.) The wide range in amounts of TC II in serum among species points out why it is necessary to account for this variability in planning studies of Cbl uptake, since presumably the Cbl attached to TC II naturally *in vivo* is functionally the equivalent of the CN [^{57}Co]Cbl attached to TC II *in vitro* as part of the determination. The "TC II" identified in chicken serum may not be TC II at all. The fractionation method used gives a small but known cross-contamination (13) and with the very large amount of non-TC II binding protein present in chicken serum, the apparent TC II in the $(NH_4)_2SO_4$ precipitate could well be a contaminant.

The reactivities of the different TC IIs in the RIA for human TC II are illustrated in Fig. 1. Only the TC II of the baboon showed definite reactivity with the antihuman TC II and the competition for antibody binding sites by baboon TC II was about a third that expected from equal amounts of human TC II.

TABLE I. COMPARTMENTS OF TC II IN DIFFERENT SPECIES AS NG CBL BOUND/ML SERUM

Species	Total UBBC[a]	Apo TC II	Holo TC II	Total TC II[b]
Man[c]	1.25 ± 0.24	1.16 ± 0.22	0.10 ± 0.05	0.92 ± 0.09
Baboon	1.56	0.48	0.84	1.32
Dog	1.34	0.85	0	0.85
Rabbit	10.98	8.66	35.80	44.46
Guinea pig	2.08	1.77	2.06	3.83
Goat	6.81	2.80	0	2.80
Horse	2.68	2.04	0.78	2.82
Pig	1.42	0.22	0	0.22
Chicken	297.50	6.19	0	6.19

[a] UBBC is unsaturated B_{12} (Cbl) binding capacity (13).

[b] Determined from bioassay of TC II fraction after conversion of all TC II to holo TC II (11).

[c] Values for 10 normal sera, means ± 1 SD, collected and studied as described (13, 14).

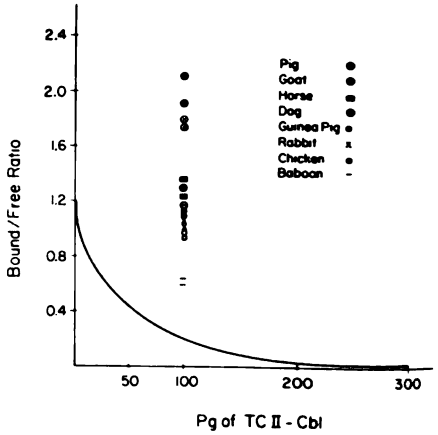

FIG. 1. RIA of the TC II from eight species by an assay for human TC II based on rabbit antibody against pure human TC II (12). The amount of material applied was adjusted to contain 100 pg of TC II. The two points shown for each TC II are the results of duplicate measurements. The line plot is the standard curve for the assay. The paired points for the standard curve were averaged for the plot.

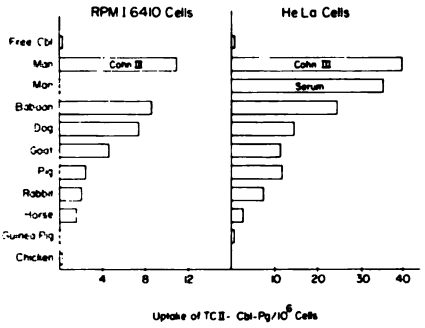

FIG. 2. The uptake of the TC II-Cbl from the sera of nine species by RPMI 6410 human lymphocytes and HeLa cells. The ratio of total TC II-Cbl to the number of cells was held constant at 500 pg per 2.5–3 × 10^6 cells. It should be noted that the measure of uptake was not of the *competitive* type as sometimes applied (2), but consisted of a *comparison* of the uptake of 500 pg of each TC II-Cbl with that 500 pg of human TC II-Cbl. The reference human TC II-Cbl was obtained from both serum and Cohn Fraction III for the HeLa cell study, but only from the latter for RPMI 6410 uptake. The values shown are means of duplicate experiments, and all forms of TC II-Cbl were examined in a single assay. Not illustrated because performed separately was the uptake of murine TC II-Cbl by HeLa cells which was 1/5 that of human TC II-Cbl but 15× that of free Cbl.

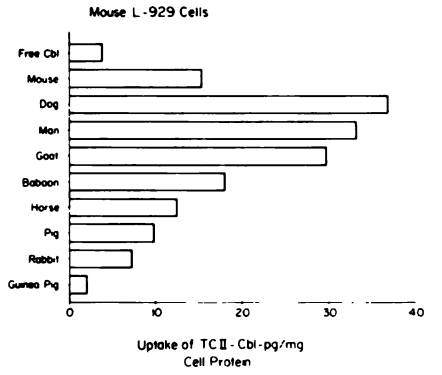

FIG. 3. The uptake of TC II-Cbl from the sera of 10 species by L-929 murine fibroblasts. The ratio of TC II-Cbl to cells was held constant at 500 pg per 3 × 10^6 cells. The values shown are means of duplicate experiments.

The uptakes of the various TC II-Cbls by RPMI 6410 human lymphocytes and HeLa cells are given in Fig. 2. As before (1), the TC II-Cbl of a primate, which most closely resembled that of man immunologically, was also closest in the ability to promote Cbl uptake by human cells.

The uptake of the same TC II-Cbls by murine fibroblasts are shown in Fig. 3. They were not equally potent in promoting Cbl uptake.

Discussion. The high degree of specificity of antihuman TC II in the reaction with TC II of species other than primates, as observed earlier, was confirmed by a completely different system of measurement. The apparent conflict between the two studies is not a serious one. Whereas by one technique (1) canine TC II did react, the reactivity was only partial (16). Neither are the present results in conflict with those of Tan and Blaisdell (3) who observed a low order of specificity between mammalian TC IIs and antibody raised against rabbit TC II. There may be specificity between two substances in one direction, as for guinea pig TC II and antibody against human TC II for example, but not necessarily in the reverse. The present study does show that the factor of immunologic specificity must be considered and measured in any studies where an attempt is made to identify TC II from one species by antibody made against TC II of another.

The varied potency of mammalian TC IIs as promoters of Cbl uptake by human cells was again demonstrated in the present study. However with the exception of the TC II of the baboon there was not the same close correlation between reaction with anti-TC II and function. The present uptake data should be considered more reliable than that of earlier studies because the uptake of the native TC II-Cbl, which varied from 0 to 35.8 ng/ml of serum, was included in the measurements. The present uptake studies were capable of showing different uptakes of an equal amount of the different TC II-Cbls. Perhaps the technique of showing immune responsiveness was incapable of detection of small gradations although in general we considered it superior to the earlier method (1). The possible relationships between immunoreactivity and function of promotion of Cbl uptake, or lack of them, may have bearing on the molecular sites of the respective activities. This is a most important question, but the present study was not designed specifically to answer it and it must be approached in another way.

Apparently murine cells are selective as

well, although the pattern of their response to TC II is different. The TC II of four species was more effective than murine TC II. This is not a new phenomenon, but is unexplained. In an earlier study (8) human TC II was possibly more effective than murine TC II in L-929 cells. Moreover, TC II from one source within an animal may be more effective than that from another (7, 8). For example, TC II-CN Cbl from dog kidney was more effective than TC II-CN Cbl from the serum of the same dog in promoting uptake by the dog liver. The form of Cbl bound to TC II which could vary in proportions among species does not seem to be a likely factor in the present study. Whereas most of the studies showing CN Cbl, OH Cbl, Me Cbl, and Ado Cbl to be equivalent in cell uptake have been conducted in microorganisms, there is one report of the equivalency of Me Cbl and CN Cbl in human systems (17).

It is difficult to compare the present studies with similar evaluations of species specificity incorporating the reticulocyte as the receiving cell (3). The originators of the reticulocyte model considered the uptake observed to consist of the primary phase only (18). Meaningful cellular uptake of Cbl requires a secondary phase as well (1) and there are circumstances where uptake begins but is never completed (2). However, species specificity may be observed at the primary level. Friedman *et al.* (19) found the affinity constant between the isolated receptor for rat TC II from human placenta to be less than 1/5 that of human TC II while bovine TC II did not bind at all. Youngdahl-Turner *et al.* (20) found rabbit TC II-Cbl to express only partial competition for the receptors of TC II-Cbl of human fibroblasts at 4°.

Summary. The reactivity between antihuman transcobalamin II (TC II) and the TC II from eight species of animals was evaluated by a radioimmunoassay for human TC II. None of the animal TC IIs reacted to the same degree as an equal amount of human TC II, although the TC II of the baboon expressed partial reactivity. The function of each TC II in promoting the uptake of Cbl was evaluated by exposure to cultured human HeLa cells and human lymphocytes. There was considerable variation in potency with some TC IIs having no effect, while others, such as that of the baboon, were almost as active as human TC II. The same species of TC II expressed varied potency for promoting uptake by murine fibroblasts as well. We have concluded that antibody against human TC II and the TC II mediated complete uptake of Cbl by human cells exhibit species specificity.

The TC II-Cbl from some species was less effective than murine TC II-Cbl or the murine L-929 system, but some forms of TC II-Cbl were more effective.

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ıtion and Characterization of Erythrocyte Receptors for Measles Virus (40669)[1]

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sles virus agglutinates monkey and ba- rythrocytes (1), but not erythrocytes of ıpecies. Hemagglutination (HA) is ef- through the interaction of the H spike rotein in the viral envelope with recep- ı the cell surface (2, 3). Little else is . about the receptor for this virus on erythrocytes or susceptible cultured xcept that *N*-acetyl neuraminic acid A) is not required for attachment. In- neuraminidase treatment of erythro- nhances their susceptibility to measles HA, and exposure of Vero cells en- slightly their susceptibility to infection eviously presented data indicated that eptor on erythrocytes is a glycoprotein can be obtained in water-soluble form ıtment of membranes with lithium diio- ylate and phenol (LIS-ph) (5). We re- ıere the characterization of simian ocyte glycoproteins and present data point to their role as the receptors for s virus.

erials and methods. Cells and virus. ells were grown to confluence in roller in minimal essential medium (MEM) mented with 10% fetal calf serum . Cells were washed twice with PBS oculated with the LEC strain of mea- rus at a multiplicity of infection of 1 cell in Hanks' balanced salt solution. ıdsorption the cells were overlaid with containing 1% FCS. After 3 days, the ere harvested and the culture fluid was d by centrifugation at 1200 *g* for 10 larified fluids were stored at −70° until or purification of virus.

ıaration of erythrocyte ghosts. Rhesus y erythrocytes were obtained from Laboratories and from the Delta Re- Primate Research Center, Covington, Louisiana. Packed erythrocytes in Alsever's citrate dextrose solution were washed and processed to hemoglobin-free membranes by the method of Dodge *et al.* (6). Hemoglobin-free ghosts were similarly prepared from sheep and human erythrocytes.

Extraction of proteins from erythrocyte ghosts. Proteins were extracted from erythrocyte ghosts according to the method described by Marchesi *et al.* (7). Briefly, lyophilized ghosts were mixed with a solution of 0.3 *M* LIS in 0.05 *M* Tris–HCl buffer, pH 7.5, at a concentration of 25 mg protein/ml. Water-soluble material was mixed with an equal volume of aqueous phenol and centrifuged at 4000 *g*. The aqueous phase was removed, dialyzed extensively against water, and lyophilized. Recovered proteins were washed with ethanol, relyophilized, and stored at 4°.

Analysis of membrane proteins by polyacrylamide gel electrophoresis (PAGE). Lyophilized erythrocyte membranes or LIS-ph-solubilized proteins (4 mg protein/ml) were mixed with water and treated with 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol (2-ME) in 0.05 *M* Tris–HCl, pH 6.8, and heated at 100° for 4 min. Particulate matter remaining in the solubilized mixture was removed by centrifugation at 2000 *g* for 10 min. Samples of approximately 100 μg protein (8) were analyzed in tube gels, using the discontinuous method (9), with either 7.5 or 9% separating gels and 3% stacking gels. Following electrophoresis, gels were stained by Coomassie blue (CB) or periodic acid Schiff (PAS) (10) methods or with resorcinol for sialic acid (11). Estimates of molecular weight were done in the same gel system, using, as standards, β-galactosidase, lactoperoxidase, bovine serum albumin (fraction V), catalase, glutamate dehydrogenase, fumarase, ovalbumin, pepsin, and trypsin.

Isolation of proteins from gels. Preparative gel electrophoresis was performed in slab gels

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using 3% stacking and 9% separating gels. A longitudinal slice with measured marks was cut from the gel and fixed and stained by the PAS method. Unstained portions of the gel corresponding to individual glycopolypeptides were excised, frozen, and minced with mortar and pestle. Gel fragments were stirred at room temperature with several changes of water and the slurry was then centrifuged to remove particulate matter. The supernatant was divided into two portions. One portion was lyophilized, resuspended in 5 ml of water, and dialyzed against 0.5 *M* Tris–HCl, pH 6.8. The other portion was dialyzed against five changes of 20% isopropanol, then dialyzed against water, lyophilized, resuspended in 5 ml of water, and finally dialyzed against Tris buffer.

Assays for hemagglutination inhibition (HI). Phosphate-buffered saline (PBS), pH 7.2, was used for dilutions and to suspend components in the assay. Serial twofold dilutions in PBS in volumes of 0.025 ml were made of LIS-ph-extracted proteins (1 mg/ml) from sheep, rhesus monkey, or human erythrocyte ghosts. Eight hemagglutinating units (HAU) of purified measles virus in 0.025 ml of PBS were added to each well, mixed, and incubated for 30 min at 37°. Twenty-five microliters of washed monkey erythrocytes was added to each well of the dilution series. The plates were incubated at 37° for 1 hr and HI titers were determined.

HI titers of individual glycosylated polypeptides of erythrocyte ghosts were determined, after elution from preparative gels and extensive dialysis against 20% isopropanol and PBS. The amount of SDS remaining in the dialyzed preparations was estimated by titering hemolytic activity compared with that of solutions of known concentrations, in order to establish the necessary controls for HI assays. Eluted glycoproteins in 0.025 ml were serially diluted and 8 HAU of virus in 0.025 ml (were serially diluted and 8 HAU of virus in 0.025 ml) was added to each dilution. Bovine serum albumin was added to the mixtures to a final concentration of 1%, followed by 0.025 ml 1% rhesus monkey erythrocytes fixed in 0.05% glutaraldehyde (12).

Adsorption of membrane glycoproteins to purified measles virus. Measles virus was purified by previously published methods (13). Twenty-five microliters of LIS-ph-extracted proteins (500 μg/0.5 ml) were mixed with ~20,000 HAU/ml of purified measles virus and incubated at 37° for 1 hr. The mixture was layered onto 1.5 ml of 20% (w/w) sucrose in PBS and centrifuged at 189,000 *g* for 45 min in the SW 50.1 rotor. The supernatant was removed and the virus pellet was suspended in PBS and recentrifuged in the type 30 rotor at 50,000 *g* for 75 min. LIS-ph-extracted proteins without virus were layered over the sucrose barrier and processed in an identical manner. The virus plus adsorbed proteins were then solubilized and analyzed by PAGE.

Results. PAGE analysis of erythrocyte ghost proteins. Rhesus monkey erythrocyte ghosts were solubilized with SDS and 2-ME and the proteins were analyzed by PAGE. Profiles of erythrocyte membrane glycopolypeptides and polypeptides stained with PAS and CB are shown in Fig. 1. Five components stained with PAS (Fig. 1A) which had approximate molecular weights, respectively, of 120K (PAS 1), 90K (PAS 2), 64K (PAS 3), 48K (PAS 4), and 33K (PAS 5). PAS 3 and 5 consistently gave a lighter stain than PAS 1, 2, or 4. The positions of bands stained with CB (Fig. 1B) did not correspond with those stained with PAS. CB bands 1 and 2 were high-molecular-weight polypeptides of ap-

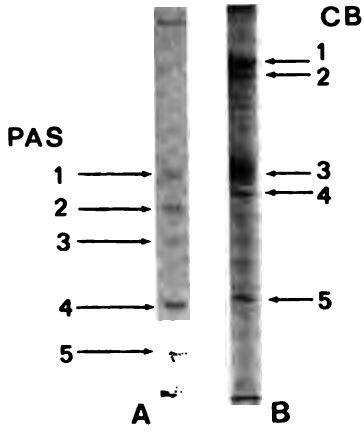
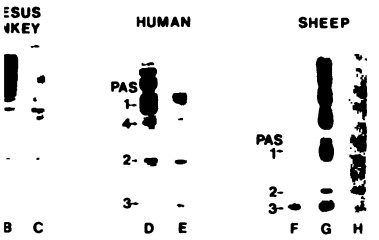


FIG. 1. PAGE analysis of membrane proteins from rhesus monkey erythrocytes. Membranes were treated with SDS and 2-ME and heated at 100° for 4 min. 100 μg of each protein was electrophoresed in SDS–disc gels. Gels A and B were stained by PAS and CB methods, respectively.

ly 280K and 240K M_r, respectively. 3 was diffuse, possibly consisting of mponents as suggested by the gra- staining. Several minor bands th CB in the regions between CB 2 4 and 5, and ahead of CB 5.

analysis of LIS-ph-extracted glyco- f erythrocyte ghosts. Proteins solu- LIS-ph treatment of rhesus mon- an, and sheep erythrocyte ghosts yzed by PAGE and stained by the iod. Additional bands were evident -ph-extracted preparations of rhe- y erythrocytes (Fig. 2B) compared een in the gels of membranes di- bilized with SDS and 2-ME (Fig. eover, in profiles of LIS-ph glyco- here was a close correspondence PAS- and CB-stained bands (not contrast to the dissimilarity be- - and PAS-stained bands of whole es (Fig. 1). All LIS-ph-extracted des were also stained by the resor- hod (11) indicating the presence of ot shown). Polypeptides of human erythrocytes were extracted and similarly. On comparison, the mul- igh-molecular-weight bands found rhesus monkey preparations were l in extracts of human and sheep



PAGE analysis of glycoproteins (PAS erythrocyte membranes and their binding to us. Rhesus monkey and sheep erythrocyte free of hemoglobin were treated with SDS nd heated at 100° and soluble proteins were , and F, respectively). Rhesus monkey, hu- eep erythrocyte membranes were also treated and the water-soluble proteins were dena- SDS and 2-ME by heating (B, D, G, respec- eins extracted with LIS-ph were mixed with us and incubated, and virus was then centri- gh a sucrose barrier. Viral proteins and eryth- brane proteins adhering to virus were solu- , H).

erythrocytes. One minor and two major bands were seen above the four glycopolypeptides (PAS 1–4) found in human erythrocyte ghosts (Fig. 2D). In LIS-ph preparations of sheep erythrocyte membranes four diffuse bands migrated behind PAS 1, 2, and 3 (Fig. 2G), the latter glycopolypeptides being found solely in ghosts (Fig. 2F). PAS 1 was enriched compared to its counterpart in ghost profiles.

Interconversion of PAS-positive polypeptides. Rhesus monkey erythrocyte membranes were solubilized with SDS and 2-ME and electrophoresed in preparative slab gels. Bands PAS 1 to 5 were located, eluted from gel slices, and dialyzed against either isopropanol or buffer as described under Materials and Methods. PAGE analysis of the eluted bands which had not been dialyzed against isopropanol yielded bands in the same position as before isolation. However, as shown in Fig. 3 bands dialyzed against isopropanol were resolved into bands of higher and lower molecular weight in addition to the original band. Eluted PAS 1 yielded not only PAS 1, 2, and 4, but also three bands migrating more slowly than PAS 1 and designated a, b, and c. PAS 2 yielded PAS 1, 2, and 4, small amounts of PAS 3 and 5, and bands b and c. PAS 3 yielded PAS 1, 2, 3, 4, and 5 and band c. Band PAS 4 gave rise to PAS 1, 2, and 4. PAS 5 yielded PAS 1 and 3, but no detectable PAS 5.

Hemagglutination inhibition of LIS-ph-extracted proteins and isolated glycopolypeptides from erythrocyte membranes. LIS-ph-extracted proteins from human, rhesus monkey, and sheep erythrocyte membranes were tested for inhibition of hemagglutination by measles virus. As shown in Table I, only LIS-ph-extracted proteins of rhesus monkey erythrocytes significantly inhibited hemagglutination, this fraction being at least 10 × more potent than similar fractions of human or sheep erythrocytes. Glycopolypeptides from rhesus monkey ghosts (PAS 1–5) each inhibited the agglutination of glutaraldehyde-fixed homologous erythrocytes.

Adsorption of LIS-ph-extracted proteins to purified measles virus. Lis-ph-extracted proteins from sheep, rhesus monkey, and human erythrocytes were mixed with purified measles virus and incubated at 37°. Following

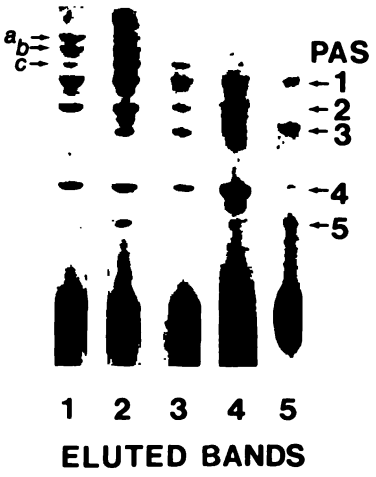


FIG. 3. Relationship between individual glycopolypeptides from membranes and those from LIS-ph-extracted material. Membrane ghosts were treated with SDS and 2-ME and electrophoresed in preparative SDS-disc slab gels. Bands PAS 1 through 5 were located and individually sliced from the gel and eluted into water. Eluates were dialyzed against 20% isopropanol and then 0.05 *M* Tris buffer. Each band preparation was reelectrophoresed (50–75 μg protein/gel) in SDS tube gels and stained with PAS. Gels 1–5 correspond to the individual band eluted from preparative gels. PAS 1 through 5 indicate the position of each PAS band from membranes.

TABLE 1. HI ACTIVITY OF ERYTHROCYTE GLYCOPROTEINS

Fraction	μg inhibiting 8 HA units measles virus
LIS-ph glycoproteins	
Human	>108
Rh Monkey	12
Sheep	>110
PAGE eluates	
Rh monkey	
PAS 1	11
PAS 2	10
PAS 3	19
PAS 4	8
PAS 5	23
Human glycophorin A	>50

incubation, the virus was centrifuged through a sucrose barrier and washed once. The virus pellets were then analyzed by PAGE. LIS-ph-extracted material alone served as control and no pellet was recovered. As shown in Fig. 2C, only two major glycopolypeptides from the total LIS-ph-extracted proteins of rhesus monkey erythrocytes (Fig. 2B) adsorbed to measles virus. The two bands corresponded in electrophoretic migration to PAS 2 and 4 of the polypeptide profile of membrane (Fig. 2A). In addition, several lightly staining bands were evident migrating above and below PAS 2.

LIS-ph-extracted proteins from human and sheep erythrocyte membranes were adsorbed with purified measles virus in similar manner. Adsorbed virus was analyzed by PAGE along with the LIS-ph-extracted proteins. Of the total human erythrocyte LIS-ph-extracted proteins (Fig. 2D) the upper, most slowly migrating portion of PAS 1 adsorbed to the virus (Fig. 2E). In addition, PAS 2 and 3 and a trace of PAS 4 adsorbed to the virus. Aggregated glycopolypeptides which migrated behind PAS 1 did not adsorb to virus under these conditions. Of the six PAS bands of sheep erythrocyte glycoproteins, only PAS 2 adsorbed to measles virus to any appreciable degree (Fig. 2H).

Discussion. The glycoproteins extracted from membranes of rhesus monkey erythrocytes inhibited measles virus-mediated agglutination of homologous erythrocytes. Bands corresponding to PAS 2 and 4 of the PAGE profile of erythrocyte ghosts were preferentially adsorbed by purified virus. Aggregated glycoproteins were not found with the viral pellet and no glycoproteins by themselves in the absence of virus passed through the sucrose barrier. These facts argue for some degree of specificity in this interaction. Data to suggest that certain of the glycoproteins in LIS-ph extracts of simian erythrocytes act as receptors in intact membranes were obtained by analysis of individual glycopolypeptides eluted from preparative PAGE. Each band (PAS 1–5) by itself inhibited agglutination. PAGE analyses of eluted glycoproteins after dialysis against isopropanol yielded in each case not only the original bands, but also aggregated forms of higher and lower molecular weights not previously present as such. The final patterns of glycopolypeptides so derived from each of the original eluted bands were all very similar to one another, except for that of PAS 5, which in contrast to the others showed the least specific activity. After dialysis, PAS 4 gave rise to PAS 1 and 2, and PAS 5 yielded PAS 1 and 3. PAS 4 and 5 may therefore be monomers which

in structure and which form correlingly diverse aggregates. Accordingly, 2 and 4 were preferentially adsorbed to ed virus and had higher specific antiactivity than PAS 5. This suggests that l and/or its derivatives serve as the chief tors for measles virus, PAS 5 having imited activity.

hough neither human nor sheep erythes are agglutinated by measles virus, le glycoproteins of either species were ically adsorbed to virus. Of the six sheep olypeptides, only one was reactive with

From the analogous human material, al components appeared to be specifiadsorbed. Most prominent was the slowrtion of the diffuse PAS 1 (glycophorin)

The two polypeptide bands with mor weights higher than glycophorin (Fig. lid not adsorb. PAS 2 and 3, however, dsorb to virus. This may be explained e fact that PAS 1 represents aggregates lower-molecular-weight glycopeptides, y PAS 2 and 3 (14) which may, therehave the same specificity for virus as the ve portion of PAS 1, and which is not perty of the components larger than 1.

pointed our earlier, receptors on sheep uman erythrocytes possibly because of considerations, are not accessible to hemagglutinin, yet certain of the glycoins solubilized from membranes are sely reactive with virus. In order to exthis apparent discrepancy, one could se that adsorption of soluble receptor protein by virus is a multiphase process, with the sheep and human material not interfere with attachment of viral o intact receptors on simian erythro-

nmary. Glycoproteins extracted in water-soluble form from rhesus monkey erythrocytes were shown to inhibit hemagglutination by measles virus. PAGE analysis of glycoproteins from erythrocyte membranes and in LIS-ph extracts showed that lower-molecular-weight glycoproteins readily formed aggregates which resisted dissociation with SDS and 2-ME at 100°. Preferential binding of isolated glycoproteins of erythrocyte membranes to purified virus was demonstrated even though all of these inhibited hemagglutination to some extent. Data presented here suggest that specific forms or configurations of membrane glycoproteins constitute receptors for measles virus.

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Cell Surface Binding Proteins for the Major Envelope Glycoprotein of Murine Leukemia Virus (40670)[1]

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The initial step in establishing a productive oncornavirus infection requires the presence of specific "receptors" on the surface of the host cell (1). Secondary factors, however, subsequent to adsorption have also been shown to influence host susceptibility, i.e., Fv-1 restriction (2, 3). The 71,000 M_r major envelope glycoprotein (gp71) of murine leukemia virus (MuLV) is required for virus–receptor interaction and subsequent infection. Interference studies utilizing purified gp71 indicate that N and B tropic viruses share the same family of receptors, whereas mouse xenotropic viruses use a different family of receptors than do ecotropic viruses (4–6). To our knowledge however, no information is presently available identifying those cell surface component(s) involved in structuring the functional receptor for oncornaviruses.

The spleen and thymus are target tissues for murine leukemia virus and viral specific sequences were detected in the DNA of these tissues in leukemic mice infected at birth with virus (7). We have previously described an *in vitro* system for studying the binding characteristics of Rauscher murine leukemia virus (R-MuLV) gp71 to cells derived from susceptible murine target tissues (8). The binding of gp71 to mouse fibroblasts in culture has also been recently described (9). In this study we have used a cleavable bifunctional chemical reagent to reversibly link R-MuLV gp71 bound to thymocyte surface polypeptides in the vicinity of the gp71 binding site. Analysis of immune precipitates of covalently linked "gp71-receptor" conplexes after reductive cleavage has been used to identify polypeptides either comprising or "nearest neighbors" to the binding site "receptor" for R-MuLV gp71.

Materials and methods. Thymic cells from 6-week-old BALB/c mice were prepared as previously described (8) and were more than 90% viable as determined by trypan dye exclusion. Cells ($1–5 \times 10^7$/ml of medium) were labeled overnight at 37° in a 5% CO_2 atmosphere on a rocker platform in leucine-free RPMI 1640 media containing 200 m*M* glutamine, [^{3}H]leucine (0.5 mCi/ml [22 Ci/mmole]) and 10% dialyzed fetal calf serum. The major envelope glycoprotein gp71 was purified as previously described (10) from freeze–thaw preparations of double-banded R-MuLV produced in JLS-V9 cells (10^{12} virus particles/ml; 2×10^8 focus-forming units/ml). The purified glycoprotein (Schiff's positive) was labeled with ^{125}I ($2–5 \times 10^4$ cpm/ng of protein) using the chloramine-T method (11) and migrated as a single band on SDS linear 7.5–25% gradient polyacrylamide gel electrophoresis (PAGE) with an estimated molecular weight of 71,000 M_r. Binding reactions contain 1×10^7 [^{3}H]leucine cells/ml and were incubated at 37° in the presence of ^{125}I-labeled gp71 in RPMI 1640 containing 1% BSA. The [^{3}H]leucine cell surface polypeptide–^{125}I-labeled gp71 bound complex was solubilized in a solution containing 0.25% NP40. Cross-linking with methyl 4-merceptobutyrimidate (MMB) was a modification of the procedure as described by Traut *et al.* (12). NP40-solubilized ^{125}I-labeled gp71 [^{3}H]leucine thymocyte surface polypeptides were reacted with MMB (3 mg/ml) for 6 min at 22° and were quenched by the addition of ethanolamine-HCl (pH 8.0) (0.14 *M* final concentration) and incubated for another 10 min. Disulfide bond formation between MMB-introduced thiol groups was initiated by the addition of hydrogen peroxide (40 m*M*) followed by a 30-min incubation at 24°. Samples were first counted in an ultra-

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rack gamma counter and then solubilized and recounted in a Searle liquid scintillation counter utilizing a computer-directed program to determine spillover. Appropriate standards of ^{125}I, ^{3}H, and ^{125}I + ^{3}H were included and ^{3}H activity then calculated. Protein was determined using the Bramhall assay (13).

Results. BALB/c thymocytes incorporated 145,000 cpm of [^{3}H]leucine per 10^6 cells (0.7 pmole/μg protein) when incubated for 10 hr at 37° in the presence of RPMI 1640 supplemented with excess glutamine (200 m*M*) and 10% fetal calf serum. The binding of gp71 was initially linear with 50% maximum binding (2.5–3.0 ng of ^{125}I-labeled gp71/10^6 cells) occurring within 30 min at 37° (8). Antibody to purified R-MuLV gp71 prevented binding and the addition of cold gp71 competitively inhibited the binding of the ^{125}I-labeled gp71 to thymocytes.

Soluble ^{125}I-labeled gp71 bound [^{3}H]leucine cell surface polypeptide complexes stripped from washed thymocytes with 0.25% NP40 were immediately introduced into reaction mixtures containing the cleavable cross-linking reagent MMB. The kinetics of the cross-linking reaction for gp71 are rapid and maximum extent of ^{125}I-labeled gp71 oligomer formation was observed after 6 min of incubation at 10 m*M* MMB. SDS–PAGE (Fig. 1) under nonreducing conditions (no β-mercaptoethanol) of the products of these reaction mixtures demonstrated the formation of high-molecular-weight oligomers containing disulfide-linked ^{125}I-labeled gp71 and [^{3}H]leucine thymocyte polypeptides migrating in the nonlinear region of the gel (fractions 5–10). Gel slices corresponding to these fractions contained a total of approximately 7000 cpm of ^{125}I and 35,000 cpm of [^{3}H]-leucine as determined by differential counting (Materials and Methods). These oligomers were completely cleaved by extensive reduction with 3% β-mercaptoethanol to monomers migrating in the region of gp71 (fractions 60–70). Data on the immunoprecipitation with various antisera of either NP40 stripped, MMB cross-linked ^{125}I-labeled gp71 preincubated with thymocytes and ^{125}I-labeled gp71 samples which were not preincubated is presented in Table I. Experiments in which unlabeled gp71 was used in

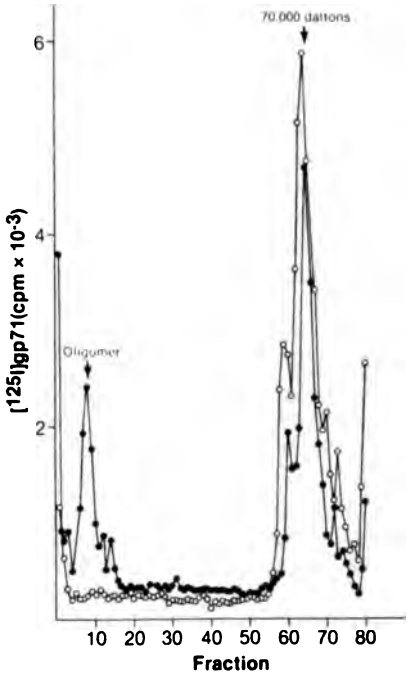


FIG. 1. SDS–Polyacrylamide gel electrophoresis of MMB-cross-linked ^{125}I-labeled gp71 oligomers under reducing (○) and nonreducing conditions (●). Samples for reductive cleavage were removed from MMB-cross-linking reactions and extensively dialyzed against 3% β-mercaptoethanol at 4° for 25 hr followed by dialysis against SPB buffer (50 m*M* Tris–Cl, pH 6.7, 1% SDS, 5% β-mercaptoethanol 10% glycerol and 0.005% bromophenol blue) and electrophoresed in 7.5–20% gradient SDS–polyacrylamide tube gels as previously described (31). Gels were cut in 1.2-mm slices and counted directly in an LKB 1207 Rackgamma M Counter.

the initial linking reaction also demonstrated the cross-linked-dependent immunoprecipitation of [^{3}H]leucine thymocyte surface proteins with gp71 antiserum. Antiserum to gp71 was preadsorbed with thymocytes (five cycles) and demonstrated to be highly specific. Postlinked ^{125}I-labeled gp71 [^{3}H]leucine thymocyte polypeptide oligomers were precipitable with gp71 antiserum (>70%) and the cross-linking reaction had little or no effect on the affinity of ^{125}I-labeled gp71 for homologous antibody. Although antisera containing H-2^d and Ia reactivity had no specificity for ^{125}I-labeled gp71 (<0.4%), the MMB cross-linking reaction did covalently bind cell surface polypeptides with determinants which were now reactive with antisera (>9% of input ^{125}I-labeled gp71). Similarly, antisera

TABLE I. IMMUNOPRECIPITATION OF MMB-CROSS-LINKED ^{125}I-LABELED GP71 THYMOCYTE SURFACE POLYPEPTIDES

Antiserum to	MMB control[a] ^{125}I-labeled gp71(cpm)			MMB-cross-linked ^{125}I-labeled gp71(cpm)		
	Input	Precipitate	% Precipitation	Input	Precipitate	% Precipitation
gp71	126,900	81,495	64.2	80,022	59,661	74.6
p30	133,098	351	0.3	72,429	12,651	17.5
p15	121,818	285	0.2	81,500	4,808	5.9
H-2^d[b]	128,331	550	0.4	75,711	6,864	9.1
IgG goat	125,325	234	0.2	74,766	393	0.5
IgG mouse	112,050	105	0.1	75,168	309	0.4

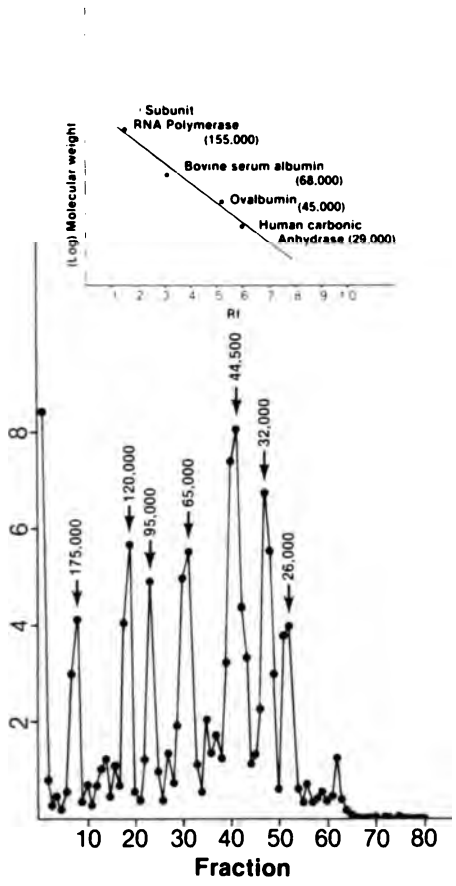
[a] Immune precipitation reaction tubes (final volume of 250 μl) contained 100-μl aliquots of either NP40-stripped, MMB-cross-linked ^{125}I-labeled gp71·thymocyte surface polypeptide oligomers or ^{125}I-labeled gp71 samples which were not preincubated with thymocytes. Antisera stock was diluted with a solution containing 0.05 *M* Tris–Cl, pH 7.2, 0.05 *M* NaCl, 0.001 *M* EDTA (TNE buffer), and 1% bovine serum albumin so that the addition of 100 μl resulted in a final antibody dilution of usually 1/200. Tubes were incubated for 2 hr at 37° and overnight at 4°. Anti-mouse IgG serum produced in goats or pigs was added at a dilution giving maximum precipitation and samples reincubated for 3 hr at 37°. Pellets (500 *g* for 15 min at 5°were washed twice in 3.0 ml of TNE buffer containing 2% BSA, 0.4% Triton X-100 followed by washing twice with phosphate-buffered saline and transferred to clean tubes. The ^{125}I cpm in the final pellet was measured in a LKB 1270 rack gamma counter.

[b] Cell-free ascites fluid served as the source of H-2 and Ia antibody and was prepared by alloimmunization of cogenic strains of mice (generous gift of D. Sachs). Antisera to viral antigens was prepared by hyperimmunization of goats with guanidine-HCl-purified viral protein.

to R-MuLV p30 and p15 precipitated 17.5 and 5.9%, respectively, of the postlinked input radioactivity (cpm). Consistent immunoprecipitation of postlinked ^{125}I-labeled gp71–cell surface polypeptide oligomers with serum containing reactivity to both H-2^d and Ia antigens was observed in four separate experiments utilizing thymocyte preparations derived from pools of 20 mice each (total 80 mice). Minimal precipitation (0.5%) was observed with either goat or mouse anti-IgG. The immune precipitates (gp71 antiserum) were reduced by dialysis against 3% β-mercaptoethanol and the molecular weights of the [^{3}H]leucine polypeptides in postlinked complexes were then estimated by SDS-PAGE. Six major [^{3}H]leucine polypeptides which migrate within the linear range of 7.5% polyacrylamide gels are seen in reduced immune precipitates (gp71 antiserum) of MMB cross-linked ^{125}I-labeled gp71·[^{3}H]leucine thymocyte surface oligomers (Fig. 2). These polypeptides correspond to estimated molecular weights of 26,000, 32,000, 45,000, 65,000, 95,000, and 120,000 M_r. The largest protein (fractions 11–13) did not migrate within the linear range of the gel and is assigned a value greater than 180,000 M_r. Washed immunoprecipitates of NP40-stripped, MMB-linked, thymocyte surface polypeptides to which no ^{125}I-labeled gp71 was added (controls) derived utilizing gp71 antiserum which was preadsorbed with receptor-positive thymocytes contained less than 500–700 cpm of [^{3}H]leucine. SDS–PAGE of these controls after reduction demonstrated low activity in the 70,000 M_r region and probably represents endogenous gp71. ^{125}I-Labeled gp71 recovered from cross-linking reactions which contained no thymocyte proteins migrated as a single band in the region of 70,000 M_r.

Discussion. The identification and characterization of cell surface polypeptides involved in structuring the functional "receptor" complex for oncornaviruses will be instrumental in further expanding our understanding of the horizontal spread of infectious virus progeny to susceptible host cells and also exploring cellular immune mechanism mediated via viral structural components (antigens) which bind to cell surface receptors and both modify and modulate cellular immune responses, i.e., destruction of virus-infected cells by cytotoxic lymphocytes.

In this study we have identified six thymocyte surface polypeptides which appear to be localized on the cell surface in the vicinity of the binding site for R-MuLV gp71. MMB has been utilized in determining the topography of *Escherichia coli* 30 S ribosomal proteins and the quaternary structures of various enzymes (12). The imidate function of the



3. 2. SDS–Polyacrylamide gel electrophoresis of ed [^{3}H]leucine polypeptides solubilized from anti- precipitates of MMB-cross-linked oligomers. Ali- were removed from MMB-cross-linking reaction res and precipitated with thymocyte-adsorbed gp71 rum as described in Table 1. Washed pellets were ed in a solution containing 3% β-mercaptoethanol NaCl and 0.01 *M* Tris–Cl, pH 7.8 (constant mixing hr at 4°) recentrifuged to remove insoluble precip- and dialyzed against SPB buffer prior to SDS– E analysis. The molecular weights were estimated semilogarithmic plots using the log of molecular it versus migration ratio of the protein standards: unit of *Escherichia coli* RNA polymerase, 155,000 ovine serum albumin 68,000 M_r, ovalbumin 45,000 nd human carbonic anhydrase 29,000 M_r.

ecule reacts with the free amino groups 71 and cell surface polypeptides and the d thiol group function introduced by the B molecule is mildly oxidized to form lfide-linked oligomers (12). From the re- ed length of the MMB molecule, (12) 15 ne inference is made that the thymocyte surface polypeptides and bound gp71 observed are spatially orientated with this distance after the initial binding reaction in order to participate in the cross-linking reaction. Initial cross-linking reactions were attempted on washed viable thymocytes (no NP-40 stripping) removed from ^{125}I-labeled gp71 binding reaction mixtures after gp71 saturation was achieved. Unfortunately, there is no specificity in the cross-linking reaction in that the imidate function of the reagent reacts with the amino group of all neighboring cell surface proteins which are within effective cross-linking distance. In our system, this resulted in extremely large insoluble oligomers which were too complex for further analysis; the utilization, however, of NP40-solubilized cell surface components in these studies although not the method of choice does provide a technically analyzable experimental system. The noncleavable bifunctional cross-linking reagent dimethyl suberimidate was also used to generate gp71·cell surface polypeptide oligomers, however the linked complexes formed were mostly insoluble and the advantage of dissociating the components of the oligmer and subsequent identification was also lost (irreversible linkage). Advantage is taken of the retained antigenicity of the gp71 molecule after the cross-linking reaction to selectively absorb out those linked complexes containing gp71 from the heterogeneous pool of thymocyte surface proteins not within distance for effective cross-linking and therefore presumably not involved in the binding of gp71. This brings up a consideration of cell membrane fluidity and cautions against absolute interpretations of the data. It is well established that membrane components diffuse freely with diffusion rates much higher relative to similar molecules in solution (14), and membrane proteins have been estimated to traverse 200 to 1400 nm/sec (15). Some of the thymocyte polypeptides observed in the cross-linked complex do not necessarily have to be a constant neighbor of the gp71 binding site but can also become companion molecules via migration (post facto) after the initial binding. Conversely, other molecules may leave the vicinity or become buried in the cell membrane after the binding reaction and become unavailable to participate in the reaction.

In addition to the molecular weight estimations, a partial identification of those thymocyte polypeptides cross-linked to gp71 was achieved using immunoprecipitation analysis before and after MMB cross-linking. The use of antisera directed against products of the major histocompatibility gene complex (H-2^d and Ia antigens) demonstrated the cross-linked dependent precipitation of ^{125}I-labeled gp71. Those thymocyte polypeptides observed in reduced gp71 immune precipitates migrating in the molecular weight region of 45,000 M_r correspond to the reported molecular weights for H-2 antigens (16). The suggestion that products of the major histocompatibility complex are involved in the interaction of viral structural components with the cell surface is not new. Fluorescent antibody experiments have demonstrated that capping of H-2 antigens resulted in the cocapping of antigens reactive with antiserum directed against envelope glycoproteins of R-MuLV (17). Viral modification of H-2 antigens specify virus infected syngeneic cells as targets of cytotoxic T-lymphocytes (18) and a hypothesis has been advanced suggesting that the H-2 molecules function as an adapter capable of interacting with viral antigens on the target cell surface (17). We have recently demonstrated the affinity of H-2 antigens for the envelope glycoprotein of R-MuLV (19) and a gene within the H-2 complex has been mapped which influences the formation of molecular complexes of the H-2^b gene product with Friend virus (FV) molecules in the plasma membrane of infected cells (20). As a consequence, inclusion of H-2^b molecules into FV, cocapping of FV, and H-2^b antigens, and target cell generation have been described. The data presented in this study also suggests that H-2 is expressed on the cell surface or moves after the initial binding event to a position near the binding site for gp71, and adds biochemical evidence supporting the physical association of gene products of the histocompatibility locus and viral antigens. Although evidence for the association of H-2 antigens and gp71 on the cell surface is considerably strong, we cannot by experimental design rule out the possibility of the reassociation of H-2 antigens and gp71 during the solubilization procedure prior to linking.

The cross-linked dependent precipitation of ^{125}I-labeled gp71·thymocyte surface polypeptides with antisera to products of the "gag" gene is surprising. Both p30 and p15 antisera precipitate approximately 17.5 and 6%, respectively, of the input gp71 only if it has been cross-linked with MMB. The expression of gag gene products in particular p30 gag precursor on the cell surface of infected (21) and normal mouse cells during embryogenesis (22) has been reported. The major polypeptide found in reduced (p30) immune precipitates of cross-linked gp71·thymocyte surface proteins migrates on SDS-PAGE in the range of 65,000–85,000 M_r (data not shown). A 65,000 M_r polypeptide is observed in the gel profile of Fig. 2 (fractions 28–32). This corresponds in the range of the molecular weight reported for the gag precursor 65,000–85,000 M_r (23) and therefore suggest possibly that viral precursor molecules are in the vicinity of the receptor for gp71.

In conclusion, the data we have presented supports previous observations that products of the major histocompatibility locus interact with viral antigens on the cell surface. Preliminary evidence is also presented which suggest that gag gene products are also localized in the vicinity of the binding site for gp71. These studies have also demonstrated that the use of *cleavable* bifunctional cross-linking reagents followed by the specific immunoprecipitation of the molecule of interest (gp71) is a valuable tool for studying the interaction of molecules on the cell surface.

Summary. The 71,000 M_r major envelope glycoprotein (gp71) of Rauscher murine leukemia virus has been shown to bind to susceptible cells *in vitro*. [^{3}H]Leucine surface polypeptides bound to ^{125}I-labeled gp71 were stripped from BALB/c thymocytes with nonionic detergent and chemically linked with the cleavable bifunctional reagent methyl 4-mercaptobutyrimidate. Soluble disulfide-linked ^{125}I-labeled gp71 bound thymocyte surface polypeptide oligomers were precipitated with adsorbed gp71 antisera and after reductive cleavage, molecular weights (SDS-PAGE) of approximately 23,000, 32,000, 45,000, 65,000, 90,000, and 170,000 M_r were seen. Experiments demonstrating the *cross-linked-dependent* immunoprecipitation of ^{125}I-

abeled gp71·thymocyte surface polypeptide
)ligomers with antiserum having reactivity to
)roducts of the major histocompatibility lo-
:us (H-2^d and Ia) and also for the gag gene
)roducts (p30 and p15) are described. The
nvolvement of these thymocyte surface pol-
/peptides in structuring the functional recep-
or for ecotropic oncornaviruses is discussed.

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Effects of Plant Fiber in Decreasing Plasma Total Cholesterol and Increasing High-Density Lipoprotein Cholesterol (40671)

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Hypercholesterolemia, a common problem in westernized societies, is a major risk factor for ischemic heart disease. Whereas the total serum cholesterol concentration is directly correlated with the prevalence of heart disease, the high-density lipoprotein (HDL) cholesterol concentration is inversely correlated with heart diesase; this suggests that high HDL concentrations may protect from vascular disease (1, 2). Many dietary maneuvers including ingestion of plant fiber lower serum total cholesterol concentrations but the influence of most of these dietary alterations on HDL cholesterol concentrations have not been delineated. Certain water-soluble plant fibers have distinct cholesterol-lowering properties in humans and in experimental animals (3–6). Our preliminary studies (7) suggest that high-fiber diets selectively lower serum total cholesterol concentrations while increasing serum HDL concentrations. Previous studies have demonstrated that fibers such as pectin (3, 4, 8), guar gum (3, 5, 9) and oats (6) lower serum total cholesterol concentrations. In this study, we have examined the effects of fiber-supplemented diets on total and HDL cholesterol concentrations in rats.

Methods. Sprague–Dawley rats were individually housed in stainless-steel cages with raised mesh floors. Rats were randomly divided into five dietary groups of 10 animals each. Food and water were fed *ad libitum.* Food intake and body weights were recorded weekly. Each group of rats were fed one of the five experimental diets containing 46.5% sucrose and 10% plant fibers for 3 weeks (Table I). The five diets were: sucrose diet with 10% cellulose, sucrose–cholesterol diet with 10% cellulose, sucrose–cholesterol–pectin diet with 10% pectin, sucrose–cholesterol–guar gum diet with 10% guar gum, and sucrose–cholesterol–oat-bran diet with 10% oat-bran fiber. The sucrose diet did not contain cholesterol or cholic acid. The other four diets contained 1% cholesterol and 0.2% cholic acid. Cholic acid was added to enhance the hypercholesterolemic effects of cholesterol feeding. At the end of a 3-week period rats were anesthetized with ether and blood was withdrawn by cardiac puncture. After the rats were killed the liver was excised, blotted to remove excess blood, and weighed. Both plasma and liver were stored at −20° until analysis.

The total lipid in liver was extracted by homogenizing the tissue with a 2:1 (v/v) chloroform:methanol mixture and washing the filtered extract with one-fifth volume of 0.9% NaCl (10). The resulting mixture separated into two phases. The weight of total lipid in the chloroform layer was measured by the gravimetric method. A measured aliquot of the chloroform layer was used for cholesterol and triglyceride determinations. The plasma high-density lipoproteins (HDL) were separated by precipitation of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) with sodium phosphotungstate-Mg^{2+} (11). A measured aliquot of the supernatant was used for HDL cholesterol determination. Liver and plasma cholesterol was measured by the method of Franey and Amador (12). Liver triglycerides were estimated colorimetrically by the Dade method (13). Plasma triglycerides were measured by the method of Levy (14).

Data were analyzed by using one way analysis of variance. When significant differences were found, Duncan's multiple range test was applied to determine which mean values were significantly different (15).

Results. Initial body weights, weight gains, and food intakes were similar among the rats fed the five experimental diets (Table II).

As expected, plasma total cholesterol, liver total lipid, and liver cholesterol concentrations were significantly higher in cholesterol-fed rats than in rats fed cholesterol-free diets.

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TABLE I. COMPOSITION OF DIETS, g/100 g

	Sucrose–Cellulose	Sucrose–Cholesterol cellulose	Sucrose–Cholesterol pectin[a]	Sucrose–Cholesterol guar gum	Sucrose–Cholesterol oat bran[b]
Carbohydrate, Total	63.1	62.5	62.5	62.5	62.5
Sucrose	46.5	46.5	46.5	46.5	46.5
Starch	16.6	16	16	16	16
Protein, Total	15.6	15	15	15	15
Casein	15.6	15	15	15	7
Oat bran[b]	—	—	—	—	8
DL-Methionine	0.3	0.3	0.3	0.3	0.3
Fat, Total	6	6	6	6	6
Cotton seed oil[c]	6	6	6	6	3.5
Oat bran[b]	—	—	—	—	2.5
Cholesterol	—	1	1	1	1
Cholic acid	—	0.2	0.2	0.2	0.2
Vitamin mixture[d]	1	1	1	1	1
Salt mixture[e]	4	4	4	4	4
Plant fiber, Total	10	10	10	10	10
Cellulose	10	10	—	—	—
Pectin	—	—	10	—	—
Guar gum	—	—	—	10	—
Oat bran[b]	—	—	—	—	10

[a] Pectin (ICN Nutrition Biochemicals, Cleveland, Ohio), molecular weight 150,000–300,000, methylation 8.2%, viscosity 6.2%.

[b] Sucrose–cholesterol–oat bran diet contained 36.5% oat bran (The Quaker Oats Company, Barrington, Ill.). Oat bran contained: protein 22.1%, fat 6.9%(20.2% saturated, 34.8% monounsaturated, and 40.6% polyunsaturated fatty acids), and fiber 27.6%.

[c] Cotton seed oil provided 26.1% saturated, 18.9% monounsaturated, and 50.7% polyunsaturated fatty acid.

[d] Vitamin Diet Fortification Mixture, ICN Nutritional Biochemicals, Cleveland, Ohio.

[e] USP XVII salt mixture (ICN Nutritional Biochemicals, Cleveland, Ohio) supplemented with zinc sulfate 0.081%.

TABLE II. EFFECTS OF FIBER-SUPPLEMENTED DIET ON BODY WEIGHT, FOOD INTAKE, AND PLASMA AND LIVER LIPID LEVELS IN CHOLESTEROL-FED RATS[a]

	Sucrose–Cellulose	Sucrose–Cholesterol cellulose	Sucrose–Cholesterol pectin	Sucrose–Cholesterol guar gum	Sucrose–Cholesterol oat bran
Initial body weight (g)	284 ± 15	292 ± 19	294 ± 14	292 ± 19	295 ± 10
Final body weight (g)	330 ± 22	354 ± 22	334 ± 23	340 ± 24	354 ± 19
Food intake (g/day)	17 ± 1	18 ± 1	17 ± 2	16 ± 2	18 ± 2
Plasma total cholesterol (mg/100 ml	74 ± 11[w]	133 ± 13[x]	84 ± 11[w]	105 ± 28[x]	113 ± 8[x]
Plasma HDL[b] cholesterol (mg/100 ml)	33.8 ± 4.6w	18.4 ± 5.2[x]	25.8 ± 7.4[x]	31.0 ± 3.1[w,x]	25.3 ± 5.0[x]
Plasma triglyceride (mg/100 ml)	107 ± 23[w,x]	126 ± 48[w]	59 ± 14[x]	62 ± 23[x]	86 ± 25[x,y]
Liver total lipid (mg/g)	33.4 ± 3.6[w]	135 ± 24[x]	56 ± 16[x]	68 ± 26[x]	93 ± 28[y]
Liver triglyceride (mg/g)	8.2 ± 2.1[w]	43.2 ± 10.8[x]	14.8 ± 4.1[w]	24.4 ± 12.4[x]	34.2 ± 11.8[y]
Liver cholesterol (mg/g)	3.5 ± 1.3[w,y]	51.1 ± 14.9[x]	13.5 ±7.3[w,y]	22.9 ± 14.0[x,y]	27.1 ± 15.3[x]

[a] Mean ± SD for 9 to 10 rats. Means in the same line with different superscript letters differ significanly ($P < 0.05$).

[b] High-density lipoproteins.

HDL cholesterol levels were significantly lower in rats fed diets containing cholesterol than those in rats that did not receive cholesterol; this has been reported previously (16).

The cholesterol-fed rats that received pectin, guar gum, or oat bran had significantly lower plasma total cholesterol concentrations than those rats that received cellulose; the lowest concentrations were observed in the pectin-treated group. On the other hand, plasma HDL cholesterol concentrations were significantly higher in the cholesterol-fed rats receiving pectin, guar gum, or oat bran than in those rats receiving cellulose. The ratios of total cholesterol: HDL cholesterol for the cholesterol-fed rats treated with pectin, guar gum, and oat bran, ranging from 3.3 to 4.5, were significantly lower than the ratio for

those rats treated with cellulose, 7.3 ($P < 0.05$).

Plasma triglyceride concentrations for cholesterol-fed rats that received pectin, guar gum, or oat bran were significantly lower than those of cholesterol-fed rats that received cellulose. These concentrations were similar among the three groups treated with pectin, guar gum, and oat bran.

Concentrations of liver total lipid, liver triglyceride, and liver cholesterol were significantly lower for cholesterol-fed rats that received pectin, guar gum, and oat bran than concentrations for rats that received cellulose. As noted for plasma total cholesterol, the pectin group had lower concentrations for all three liver lipids than did the other cholesterol-treated groups.

Discussion. Pectin, guar gum, or oat-bran administration attenuate the accumulation of cholesterol in plasma and liver of cholesterol-fed rats (Table II). Similar observations have been reported previously when pectin (4), guar gum (9), or oats (6) were added to the diet of cholesterol-fed rats. Our observations are of interest because these three plant fibers were accompanied by higher plasma HDL cholesterol values than observed in cellulose-fed rats. Consequently, rats fed pectin, guar gum, or oat bran had significantly lower plasma total cholesterol: HDL cholesterol ratios than the cellulose-fed rats.

Many studies (see Ref. (7)) have documented that plant-fiber feeding is associated with reductions in serum cholesterol concentrations in humans and in animals. However, most studies (see Ref. (7)) have failed to demonstrate an effect of plant-fiber feeding on serum triglyceride concentrations. Under the experimental conditions that we used, a significant reduction in plasma triglycerides was observed in rats fed pectin, guar gum, or oat bran; liver triglyceride concentrations also were significantly lower in rats fed these three fibers than in cellulose-fed rats. When persons with diabetes are fed high-fiber diets containing a variety of plant fibers from natural foods, we have observed a significant reduction in serum triglyceride concentrations (7, 17). Further studies are required to delineate the effects of plant fiber on serum triglyceride concentrations.

Water-soluble plant fibers such as pectin and guar gum appear to have greater cholesterol-lowering properties than do water-insoluble fibers such as cellulose or bagasse (3). Oat bran was selected for these studies because approximately one-third of the plant fiber of oat bran is the water-soluble oat gum (18). These water-soluble fibers may lower serum cholesterol concentration by increasing fecal loss of bile acids (4) or by other mechanisms (7). The physiologic processes by which the soluble fibers selectively lower serum total cholesterol values but increase the HDL cholesterol values have not been examined. Durrington and colleagues (8) observed that the reduction in total serum cholesterol when normal subjects were fed pectin for a 3-week period was largely related to a reduction in LDL-cholesterol concentrations.

Therapeutic maneuvers which selectively lower total or LDL-cholesterol levels while increasing HDL-cholesterol levels will be extremely important in the management of hypercholesterolemia. These therapeutic measures may also be indicated for other patients at high risk for coronary artery disease. High-density lipoproteins (HDLs) appear to exert an antiatherogenic effect in humans (1, 2). Recent studies have reported a significant negative correlation between HDL cholesterol and ischemic heart disease (1). HDLs bind the cholesterol released from peripheral tissues such as blood vessels and, also, transport cholesterol to the liver for catabolism (1). A reduction in plasma HDLs may impair the normal clearance of cholesterol from arterial walls and thereby accelerate the development of atherosclerosis (1). *In vitro* studies demonstrate that HDLs promote the elimination of cholesterol from atherosclerotic arterial tissues (19). HDLs may also prevent low-density lipoproteins (LDLs) from entering endothelial cells (20). Enger and colleagues (21) have postulated that alterations in the molar ratio of HDLs to LDLs may disturb the balance between the uptake of cholesterol by arterial wall and its clearance from the arterial wall. Low concentrations of HDLs permit the uptake of LDLs and the release of cholesterol into tissues. Higher concentrations of HDLs reduce the uptake of LDLs and the internalization of cholesterol.

In the light of recent studies suggesting that high concentrations of HDLs exert an an-

tiatherogenic effect, our findings that soluble fibers raise the concentrations of plasma HDL cholesterol while decreasing the plasma total cholesterol concentration in cholesterol-fed animals suggest that soluble plant fibers may be beneficial in the dietary management of hypercholesterolemia.

Summary. Cholesterol-fed rats which received diets supplemented with 10% pectin, guar gum, or oat bran fiber showed a reduced accumulation of cholesterol and triglyceride in both plasma and the liver. In contrast, an increase in the plasma high-density lipoprotein cholesterol levels of these same rats was noted. This study indicates that certain plant fibers selectively lower plasma total cholesterol while raising high-density lipoprotein cholesterol levels.

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Cytoplasmic and Nuclear Estradiol Receptor in the Corpus Luteum of the Pseudopregnant Rabbit (40672)

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Several laboratories have demonstrated that estradiol is the principal luteotropin in the corpus luteum of the rabbit (1–3). As expected, specific estradiol receptors are present in such an estrogen-responsive tissue (4). The pattern of luteal cytosol receptor activity through early and late pseudopregnancy has been reported by several investigators. Estradiol receptor activity was reported to rise from Day 4 to a maximum on Day 12 of pseudopregnancy, declining toward Day 16 and then falling abruptly as pseudopregnancy ends at Days 16–18 (5). The initial appearance of estrogen receptor in the rabbit corpus luteum was the subject of two recently published studies. Mills and Osteen (6) reported elevated levels of receptors on Day 4 of pseudopregnancy indicating that receptor levels are elevated at least 24 hr before the tissue becomes estrogen dependent on Days 5–6 (7). On the other hand, Miller and Keyes (8) concluded that significant levels of estrogen receptor appear only coincidently with the onset of estrogen dependence on Days 5–6.

The present study was performed to examine both cytoplasmic and nuclear estrogen receptor activity in the rabbit corpus luteum over the early stages of pseudopregnancy. Specifically, the experiments sought to confirm the previous findings of a significant rise in cytosol binding on Day 4 of pseudopregnancy. Furthermore, the levels of nuclear receptor over the first 6 days of pseudopregnancy were examined. The results of these experiments show that the receptor is present in the nucleus after estrogen administration due to translocation from the cytoplasm.

Materials and Methods. Animals and treatment. Sexually mature, white female rabbits of the New Zealand strain weighing 4–5 kg were used. Animals were housed in individual cages with compressed food and water available *ad libitum*. Pseudopregnancy was induced in these animals by a single i.v. injection of 100 IU human chorionic gonadotropin, hCG (APL-Ayerst). In some of the studies, rabbits on Days 2, 4, or 6 of pseudopregnancy were selected in pairs with one receiving a single i.v. injection of 100 μg estradiol suspended in 1 ml of physiological saline. The animal receiving the estradiol was sacrificed 30 min after injection. The remaining rabbit in the pair received no injection. Rabbits were sacrificed by cervical dislocation, and the ovaries were quickly removed and placed in ice-cold saline for transportation to the laboratory.

Steroids. [2,4,6,7-^{3}H]Estradiol (sp. act. 91.3 Ci/mmole) was purchased from New England Nuclear Corporation. The purity of this steroid was checked prior to use using either paper or column chromatography. Purification was accomplished where indicated. Nonradioactive steroid was obtained from Schwarz/Mann.

Tissue preparation. The following procedures were completed simultaneously for the corpora lutea from all rabbits whether estrogen treated or untreated. In the laboratory under ×7 magnification, clearly discernible corpora lutea were dissected out and cleaned of adhering interstitial tissue. All procedures from this stage to completion of the binding assay were performed at 0–4°. Corpora lutea were counted, weighed, and combined with sufficient Tris–EDTA buffer (0.0015 *M* disodium EDTA and 0.01 *M* Tris, pH 7.4) to give a final concentration of 20 mg corpus luteum/ml. The tissues were homogenized in a sintered glass homogenizer and then centrifuged at 800*g* for 10 min. The supernatant from this low speed spin was then centrifuged at 150,000*g* for 40 min in a Beckman L2-65B centrifuge. The supernatant fraction (cytosol) was decanted and used to assess cytoplasmic estradiol binding activity as described below. The low-speed pellet from the 800*g* centrifugation was resuspended in Tris–EDTA buffer and utilized in the determination of nuclear estrogen receptor as described below.

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ermination of estradiol binding in the l. Estrogen binding in the cytosol was ıred by the method of Cidlowski and oon (9) and Abney (10) and as previ- described (6). Briefly, the technique was llows: 200-μl aliquots of cytosol were l to prepared glass tubes containing oncentrations of [^{3}H]estradiol ranging 170,000 to 10,000 dpm/tube (758 to 45 /tube) in a final volume of 1 ml of Tris- A buffer. A second series of tubes con- l, in addition, a 100-fold *M* excess of idioactive estradiol which gave an esti- of nonspecific steroid binding. The non- ic binding values were eventually sub- d from total binding to yield specific liol receptor activity. After an 18-hr in- ion at 4°, the estrogen which was not l to the cytoplasmic receptor was re- d by the addition of 1 ml of a suspension ı contained 0.3% activated charcoal te A previously washed to remove fines) .03% dextran (Nutritional Biochemical ıany) in Tris–EDTA buffer. Immedi- after addition of the charcoal, the con- of the tubes were mixed and then incu- for 15 min in an ice bath. The charcoal hen separated centrifugally and the su- tant fractions transferred to counting for determination of radioactivity by scintillation spectrometry. The results se counts were used to determine the ation constant (K_a) and the number of liol binding sites/mg corpus luteum ac- ıg to the method of Woosley and Mul- (11). In some studies, there was insuf- t tissue available from the rabbit ovaries nplete analysis at all five steroid con- ıtions. In these cases, the binding results ısed on fewer points.

ermination of binding of estradiol in the ır fraction. Nuclear binding sites were ified by the exchange method described ark *et al.* (12) with some modification on preliminary studies. The pellet re- g from the low-speed (800*g*) centrifu- ı of the homogenate was washed three with 3 ml Tris–EDTA buffer followed time by centrifugation at 800*g* for 10 The resulting washed, crude, nuclear ration was combined with sufficient to yield a concentration equivalent to luteal wet wt/ml buffer.

ɔrder to determine the optimal condi- tions under which to perform the exchange assay, corpora lutea were collected 6 days after hCG injection and 30 min following estradiol injection. Four-hundred-microliter aliquots of the resulting nuclear preparation were incubated at 37° for 15, 30, 60, and 90 min with [^{3}H]estradiol (486 fmoles/tube) with and without a 100-fold *M* excess of nonradioactive estradiol. Following incubation, the nuclear pellets were washed three times with 3 ml buffer followed by centrifugation at 800*g* to remove unbound [^{3}H]estradiol. Two milliliters of ethanol was then added to each sample in order to extract the bound [^{3}H]-estradiol and the ethanolic extract transferred to counting vials for determination of ^{3}H content once the solvent had been evaporated. Tritium was quantified in a Beckman LS-250 liquid scintillation spectrometer with external quench correction.

A second preliminary study was designed to show that with increasing amounts of the nuclear preparation, a proportional increase in the binding of [^{3}H]estradiol could be demonstrated. The 200-, 400-, and 800-μl aliquots of crude nuclear preparation were incubated for 60 min at 37° in the presence of [^{3}H]-estradiol (486 fmoles/ml) with and without a 100-fold *M* excess of nonradioactive estradiol. At the end of the incubation, the nuclear preparations were washed and [^{3}H]estradiol binding was determined as described above.

In a third preliminary study, 400-μl aliquots of the nuclear preparation were incubated with [^{3}H]estradiol in the presence of a 100-fold *M* excess of estradiol, progesterone, 20α-dihydroprogesterone (20α-hydroxy-4-pregnen-3-one), or testosterone for 60 min at 37°. Following incubation, the ^{3}H binding was determined as described above.

In a final initial experimental series, the association constant (K_a) for the nuclear binding was calculated in the following manner: 400-μl aliquots of the nuclear suspension were transferred to prepared glass tubes containing four concentrations of [^{3}H]estradiol ranging from 775 to 84 fmoles/tube. Another set of tubes contained a 100-fold *M* excess of nonradioactive estradiol to give an estimate of nonspecific steroid binding. The tubes were incubated at 37° for 60 min and binding was analyzed as previously described. The kinetic parameters were calculated according to the direct linear plot method of Woosley

and Muldoon (11).

Expression of results. Estradiol binding in the cytosol and nuclear fractions is expressed as fmole bound/mg corpus luteum. Some results are presented as mean ± SE of all determinations on corpora lutea of the same group from the same day after hCG injection. The results have been analyzed statistically using linear regression analysis and Students' *t* test.

Results. The first 6 days of pseudopregnancy are marked by rapid growth of the corpus luteum in the rabbit. As demonstrated in Fig. 1, luteal weights virtually triple over this interval with 4.0 ± 0.2 mg/CL on Day 2, 8.0 ± 0.4 mg/CL on Day 4, and 11.6 ± 0.4 mg/CL on the 6th day after hCG injection. Because of this marked change in luteal weight, it was necessary to correct all receptor measurements for this increase in weight of the corpora lutea. This has been accomplished by expressing the results as fmole [^{3}H]estradiol bound/mg corpus luteum. However, this method may slightly underestimate the nuclear receptor concentration since a small increase in the receptor might be obscured by the accompanying increase in luteal weight.

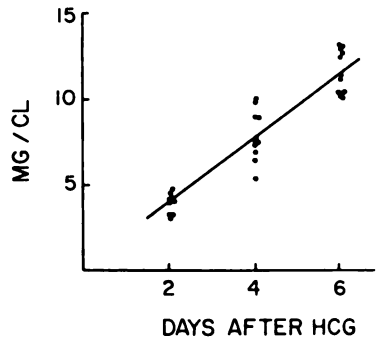

FIG. 1. The weights of corpora lutea collected 2, 4, and 6 days after a single injection of hCG. Each point is the mean weight of at least eight corpora lutea. The weight increase is linear over the 6 days ($r = 0.94$, $P < 0.05$). 100 I U hCG caused an average of 10.7 ± 0.43 ovulations/rabbit.

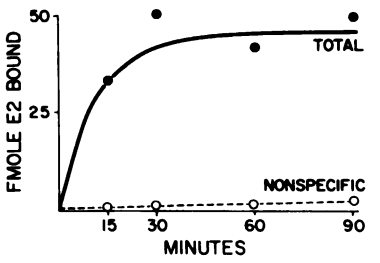

FIG. 2. The effect of duration of incubation at 37° on the binding of [^{3}H]estradiol by receptors in a nuclear fraction prepared from rabbit corpora lutea 6 days after hCG injection and 30 min after injection of estradiol. Nonspecific binding is the [^{3}H]estradiol bound in the presence of a 100-fold *M* excess of nonradioactive estradiol. Each point represents an individual determination of binding activity.

The results in Fig. 2 demonstrate that incubation of luteal nuclei for periods of 15 to 90 min at 37° does not result in any decline in estrogen binding activity. Accordingly, an incubation period of 60 min was selected for all subsequent exchange assays.

The binding activity in the nuclei of corpora lutea proves to be specific to estradiol (Fig. 3). No significant inhibition of [^{3}H]estradiol binding occurred in the presence of a 100-fold *M* excess of progesterone, 20α-dihydroprogesterone, or testosterone; these hormones were selected as representative steroids which are present in elevated concentration in rabbit corpora lutea (13) and blood (14).

Using an incubation period of 60 min, a linear relationship between the amount of luteal nuclear preparation and exchangeable estradiol binding activity can be demonstrated (Fig. 4). The data in this figure also establish that nonspecific binding activity remains low (<4% of the total activity) during the 60-min incubation.

Having established the appropriate conditions for the measurement of nuclear receptor activity in the rabbit corpus luteum a study was performed to determine how quickly after estradiol injection the cytoplasmic receptor was depleted. The results, depicted in Fig. 5, show that within 30 min after injection, cytosol receptor levels are nearly 0 and little recovery is noted by 60 min after injection. Nuclear estrogen receptors have the opposite pattern with maximal levels present at 30 min postinjection and little decline from this level by 60 min. Since depletion appears to be total by 30 min, this interval between estrogen injection and sacrifice of the rabbits was selected for all subsequent studies.

Kinetic analysis proves that the nuclear estrogen receptor in the rabbit corpus luteum is of high affinity and low capacity. The association constant (K_a) was found to be 3×10^9 *M* before estradiol injection and 3.3 ×

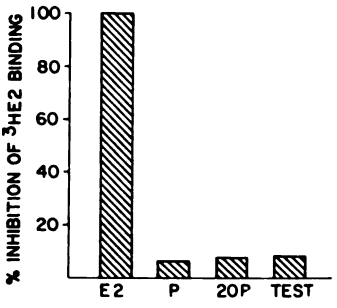

FIG. 3. The inhibition of [^{3}H]estradiol binding during the incubation of a nuclei preparation in the presence of a 100-fold *M* excess of estradiol (E2), progesterone (P), 20αdihydroprogesterone (20P), or testosterone (TEST). The inhibition of [^{3}H]estradiol binding by nonradioactive estradiol was arbitrarily set at 100% and the inhibition caused by the other steroids compared to it. The nuclei were prepared from corpora lutea 6 days after hCG injection and 30 min after estradiol administration. Each bar represents the average of two separate determinations.

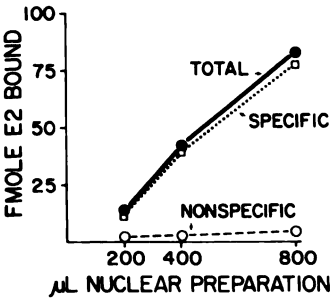

FIG. 4. The relationship between the quantity of luteal nuclear preparation and the amount of [^{3}H]estradiol bound. The 200-, 400-, and 800-μl aliquots nuclear preparation from corpora lutea collected 6 days after hCG injection and 30 min after estradiol injection were incubated alone or in the presence of a 100-fold *M* excess of nonradioactive estradiol. Specific binding is the difference between total binding and nonspecific binding. Each point represents an individual determination of binding activity.

10^9 *M* at 30 min following injection of 100 μg estradiol. In a recent preliminary communication, Yuh and Keyes (15) reported the existence of two classes of nuclear binding sites: A high-affinity site with a K_a of 7×10^9 *M* with a lower-affinity binding component of $K_a = 2 \times 10^7$ *M*. The value of 3×10^9 *M* for the association constant in the present study compares very favorably with the K_a for the high-affinity site as reported by Yuh and Keyes (15).

A portion of the results in Fig. 6 confirm findings previously published from this laboratory (6). As was reported, by Day 4 after hCG, levels of cytoplasmic estrogen receptor in animals not receiving estradiol injection are significantly increased over Day 2 levels (Day 2: 0.40 ± 0.09 fmole estradiol bound/mg CL; Day 4: 2.88 ± 0.79 fmole/mg CL, $P < 0.05$). The increase continues with even greater receptor at Day 6 (8.99 ± 2.83 fmole/mg CL). Despite this dramatic rise in cytosol receptor activity, nuclear receptor activity in the corpora lutea of rabbits not injected with

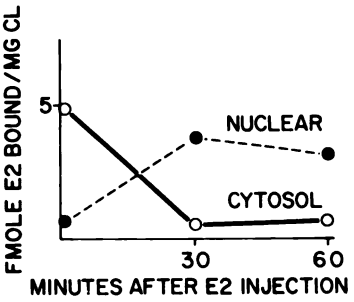

FIG. 5. The effect of time after estradiol injection on the levels of estradiol receptor in the cytosol and nuclear fractions prepared from corpora lutea 6 days after hCG injection. Each point represents an individual determination of receptor activity.

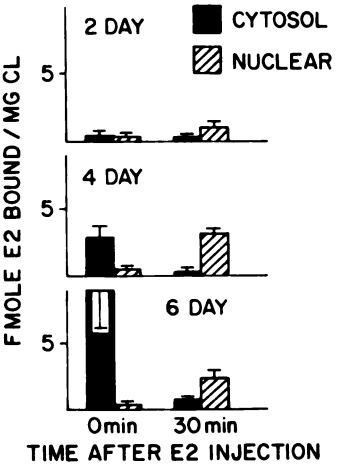

FIG. 6. Estradiol receptor activity in the cytosol and nuclear fractions of corpora lutea collected 2, 4, and 6 days after hCG injection and before (0 min) and 30 min after estradiol injection. Each bar represents the mean of three to five determinations with brackets equaling 1 SE. Solid bars—cytosol receptors, hatched bars—nuclear receptors.

estradiol changed little over the period of Days 2, 4, and 6 of pseudopregnancy. Figure 6 also demonstrates that cytoplasmic receptor disappears by 30 min after the intravenous injection of 100 μg of estradiol; on Days 2, 4, and 6 of pseudopregnancy, the estrogen injection leads to a sharp decline in levels of cytosol receptor and a concomitant increase in nuclear binding activity.

Discussion. The results of these experiments are in full agreement with the previously published finding that luteal cytoplasmic estradiol receptor increases by the 4th day of pseudopregnancy (6). The data presented in Fig. 6 show significant estrogen binding on Day 4 as compared to Day 2 after hCG ($P < 0.05$). The increase continues with elevated cytoplasmic receptor on Day 6 as well (Fig. 6). The finding of elevated cytosol receptor on Day 4 supports our previous conclusion that the receptor is increased at least 24 hr before the onset of luteal estrogen dependence reported to occur between Days 5 and 6 (7, 8). However, Miller and Keyes (8) arrive at a different conclusion in their studies. These authors feel that the onset of estrogen dependence is coincident with (and possibly due to) the appearance of estradiol receptor on the 5th day of pseudopregnancy. However, this conclusion is not fully supported by their published results; examination of Fig. 4 in their study (8) reveals a discernible increase in cytoplasmic receptor well before the onset of estrogen dependence.

Miller and Keyes (8) measured nuclear receptor activity on Days 3 through 6 of pseudopregnancy but found little change in activity except for a transient rise on the 5th day. Since the estrogen receptor is present in the nucleus only by virtue of being translocated from the cytoplasm, the elevation of nuclear receptor on Day 5 can only follow increased secretion of estradiol on the 5th day. Hilliard and Eaton (16) reported an elevation in estrogen secretion on Day 4 of pregnancy, while Challis *et al.* (17) found an increase on Day 6 of pregnancy although levels were not measured on Days 4 or 5. However, despite this apparently estrogen-mediated increase in nuclear receptor, Miller and Keyes (8) did not report the expected concomitant decline in cytosol receptor in early pseudopregnancy. In the present study, there was no measurable change through Day 6 in nuclear receptor in the corpora lutea of rabbits which did not receive exogenous estradiol although marked increases were observed in cytosol receptor over this period (Fig. 6). Possibly the estrogen secreted on the 4th and 6th day of pseudopregnancy was insufficient to cause the extensive translocation of receptor into the nucleus which follows injection of 100 μg estradiol (Figs. 5 and 6).

The studies reported here demonstrate the presence of a luteal estradiol receptor which can be translocated from the cytoplasm into the nucleus. The levels of both the nuclear and cytoplasmic receptor have been followed as the corpus luteum develops through the first 6 days after injection of an ovulatory dose of hCG. The results support the contention that the appearance of an authentic, translocatable estrogen receptor is a normal, programmed event in the sequence of luteal development and occurs prior to the onset of estrogen dependence in the rabbit corpus luteum.

Summary. Estradiol binding activity has been measured in the rabbit corpus luteum on Days 2, 4, and 6 of pseudopregnancy. The results show that receptor activity is significantly greater on Day 4 than on Day 2 with continued increases on Day 6. In addition, the estradiol receptor activity is translocated into the nucleus within 30 min of injection of 100 μg of estradiol. Appreciable receptor translocation is observed following estradiol injection on Days 2, 4, and 6 of pseudopregnancy. It is concluded that estradiol binding activity in the rabbit corpus luteum is due to an authentic estradiol receptor and that a marked increase in this activity occurs by the 4th day of pseudopregnancy.

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Evaluation of Anti-inflammatory Drugs Based upon the Inhibition of Matrix-Induced Ornithine Decarboxylase Activity during Connective Tissue Proliferation (40673)

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Subcutaneous implantation of demineralized bone matrix into allogeneic recipient rats initiates a series of interconnected cellular events resulting in differentiation of new bone (1–4). One of the early events in this sequential development is a local proliferation of mesenchymal cells in the vicinity of the implanted matrix (3). This is characterized by a sharp increase in the activity of ornithine decarboxylase (ODC; EC 4.1.1.17) 3 days after implantation (5). During the course of our studies on the effect of glucocorticoids and adrenalectomy on the process of endochondral bone differentiation we found that glucocorticoids either injected subcutaneously or implanted locally with the matrix powder inhibited the increase in ODC activity. The inhibitory effects were specific for glucocorticoids and were restricted to matrix-induced plaques (6, 7). Thus it seemed reasonable to explore the potential of this *in vivo* system of connective tissue proliferation as a method to evaluate the antiinflammatory potency of a variety of steroidal and nonsteroidal drugs. The present study describes the influence of various steroidal and nonsteroidal antiinflammatory agents on the matrix-induced ornithine decarboxylase activity.

Materials and methods. Demineralized bone matrix powder with a particle size between 74–420 μm was implanted subcutaneously in bilateral sites in the thoracic region of 50 to 80-g male rats of Long–Evans strain (1, 4). The drugs were mixed with matrix powder and implanted locally at doses ranging from 0.1 to 10 mg per rat as described in Table I. The day of implantation was designated as Day 0. Individual plaques were harvested on Day 3 after implantation and homogenized in ice-cold buffer containing 50 m*M* Tris–HCl, 5 m*M* dithiothreitol, and 1 m*M* EDTA at pH 7.2. The homogenates were centrifuged at 30,000 *g* for 30 min at 4° and ODC activity and protein were assayed in aliquots of the supernatant fractions according to Jänne and Williams-Ashman (8) and Lowry *et al.* (9), respectively, as previously described (5, 7). ODC activity was expressed as pmole of $^{14}CO_2$ released per hour per milligram of protein. The results were evaluated using the Student's *t* test.

L-[1-^{14}C]Ornithine was obtained from New England Nuclear. Corticosterone, hydrocortisone, triamcinolone, prednisolone, betamethasone, and acetyl salicylic acid (aspirin) were purchased from Sigma Chemical Company. The following compounds were generous gifts: dexamethasone and indomethacin (Merck, Sharp and Dohme), flucinolone acetonide and naproxane (Syntex Laboratories Inc.), meclofenamic acid (Warner-Lambert, Co.), chloroquine diphosphate (Sterling Winthrop Research Institute), ibuprofane (Upjohn Co.), and fenoprofane calcium (Lilly Laboratories).

Results. The results of the studies are summarized in Table I. Local implantation of steroidal antiinflammatory compounds produced different degrees of inhibition of ODC activity which varied with the structure of the compound. Synthetic fluorinated steroids such as dexamethasone, betamethasone, or flucinolone acetonide exhibited the highest inhibition of ODC activity at comparable doses. Dexamethasone also had an inhibitory effect at a much lower dose (0.1 mg). Hydrocortisone at a dose of 1 mg per rat had a minimal effect on ODC activity. Prednisolone and triamcinolone at the same dose were more potent inhibitors than either hydrocor-

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TABLE I. INFLUENCE OF STEROIDAL AND NONSTEROIDAL AGENTS ON MATRIX-INDUCED ORNITHINE DECARBOXYLASE ACTIVITY[a]

Drug	Dose (mg/rat)	ODC activity pmol $^{14}CO_2$/mg protein ± SE	*P* values	Percentage of activity
Control		550 ± 58	—	100
Steroids				
Hydrocortisone	1.0	381 ± 30	< 0.02	69
Corticosterone	1.0	346 ± 32	< 0.001	63
Prednisolone	1.0	305 ± 24	< 0.001	55
Triamcinolone	1.0	234 ± 32	< 0.001	42
Dexamethasone	0.1	193 ± 61	< 0.001	35
Dexamethasone	1.0	91 ± 32	< 0.001	16
Betamethasone	1.0	80 ± 8	< 0.001	15
Flucinolone acetonide	1.0	19 ± 8	< 0.001	3
Nonsteroids				
Indomethacin	1.0	22 ± 5	< 0.001	4
Meclofenamic acid	1.0	302 ± 21	< 0.001	55
Acetyl salicylic acid	1.0	573 ± 45	NS	104
Acetyl salicylic acid	10.0	561 ± 54	NS	102
Ibuprofane	1.0	673 ± 48	NS	122
Ibuprofane	5.0	431 ± 31	0.05	78
Fenoprofane	1.0	456 ± 33	NS	83
Fenoprofane	5.0	441 ± 79	NS	80
Naproxane	1.0	539 ± 22	NS	98
Chloroquine	1.0	541 ± 37	NS	98

[a] Six plaques from three rats were used in each experiment. NS, not significant at $P < 0.05$ level.

tisone or corticosterone. Among the nonsteroidal antiinflammatory agents examined indomethacin produced the highest inhibitory effect on ODC activity at a dose of 1 mg/rat, while meclofenamic acid produced only a 45% inhibition at the same dose. However, ibuprofane at this same dose had no significant effect on ODC activity whereas at a higher dose (5 mg) it exhibited a marginal inhibition ($P < 0.05$). On the other hand fenoprofane at two dose levels exhibited a small but insignificant inhibition of ODC activity. Chloroquine and naproxane at the dose levels tested did not influence ODC activity nor did acetyl salicylic acid at a dose of 1 or 10 mg/rat.

Discussion. The foregoing results suggest that different antiinflammatory drugs differ in their ability to inhibit ODC activity during matrix-induced mesenchymal cell proliferation. Connective tissue proliferation is one of the late events in the inflammatory reaction (10). Ornithine decarboxylase activity is a convenient marker for tissue proliferation and has been correlated with [^{3}H]thymidine incorporation (11–14).

Evaluation of antiinflammatory agents is usually limited to a few experimental models such as inhibition of erythema, edema, and granuloma confined to early phases of inflammatory reaction (10). In recent years several *in vitro* models have been developed which permit the evaluation of antiinflammatory drugs (10, 15–17). These include inhibition of prostaglandin synthetase activity (17), inhibition of *in vitro* release of lysosomal enzymes (18–19), and competitive binding of nonsteroidal antiinflammatory agents to glucocorticoid receptors (20). Since antiinflammatory drugs are widely used therapeutically in inflammatory connective tissue disorders (21, 22), it may be useful to have an *in vivo* method based on connective tissue proliferation as used in the present investigation. Gryglewski (10) has emphasized the importance of concurrent testing in several models, to predict clinical effectiveness. Therefore, the present system which simulates a late event in the inflammatory reaction such as tissue proliferation may be advantageous for the assay of antiinflammatory agents.

Although the precise mechanisms underlying the increase in ODC activity during mesenchymal cell proliferation are not known it is likely that antiinflammatory agents inhibit this response directly. Previous

experiments have eliminated the possible production of a putative inhibitor of ODC activity by glucocorticoids. Further the enzymatic activity could not be suppressed by *in vitro* addition of 5 m*M* dexamethasone (7). Recently glucocorticoids have also been shown to suppress polyamine biosynthesis and ornithine decarboxylase activity in other systems such as in psoriatic epidermis (30), spleen, and thymus (24). It is noteworthy that in the present experiment the magnitude of inhibition of ODC by steroidal antiinflammatory agents appears to be related to their potency in other assays (22, 25). In the present study dexamethasone, betamethasone, and flucinolone acetonide exhibited the greatest inhibition of ODC activity compared to equivalent doses of hydrocortisone or corticosterone. On the other hand, among nonsteroidal drugs indomethacin, meclofenamic acid, and to some extent ibuprofane were effective inhibitors of the increase in ODC activity. Aspirin, a potent inhibitor of prostaglandin synthetase (17) was ineffective even at a dose of 10 mg per rat which supports the concept that different antiinflammatory compounds may not be equally effective in every experimental system. Further, it should be emphasized that the effective potency of a drug at any time is dependent upon its solubility characteristics, possible metabolic activation, and its biological as well as plasma half-life (25). Thus a compound which may be highly effective immediately after *in vivo* administration may not be persistently as potent during the later phases. This may be the case with nonsteroidal antiinflammatory agents utilized in the present study. These considerations are of importance in the choice of drugs for long-term therapy of chronic inflammatory connective tissue disorders.

In conclusion, the bone matrix-induced elevation of ornithine decarboxylase activity during mesenchymal cell proliferation is potentially a useful model to evaluate the efficacy of steroidal and nonsteroidal antiinflammatory drugs.

Summary. The ability of several steroidal and nonsteroidal antiinflammatory agents to locally inhibit bone matrix-induced ornithine decarboxylase activity was examined. All of the glucocorticoids tested had an inhibitory effect on ODC activity. The synthetic glucocorticoids dexamethasone, flucinolone acetonide, and betamethasone were most effective in inhibiting ODC activity whereas cortisol and corticosterone had minimal inhibitory effects. The effects of the glucocorticoids on ODC activity was correlated with their known antiinflammatory potency in other systems. Nonsteroidal drugs had a varying response in this system. Indomethacin and meclofenamic acid were potent inhibitors of ODC activity whereas acetyl salicylic acid, fenoprofane, and noproxane were ineffective in doses examined.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 324-327 (1979)

Effect of Prostaglandin I_2 on Ovine Maternal and Fetal Adrenal Blood Flows (40674)[1]

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The fetal adrenal has been shown to be instrumental in the initiation of labor in several species (1). The maternal and fetal adrenals have also been shown to be sensitive to vasoactive agents (2–4). Tripp *et al.* (3) reported an increase in fetal adrenal blood flow with prostaglandin E_1 infusion and Flack *et al.* (5) reported an increase in corticosteroidogenesis after prostaglandin stimulation. There are no studies of the response of maternal and fetal adrenals to the same agent. Prostaglandin I_2 is a powerful vasodilator and does not appear to be destroyed by the pulmonary vasculature (6). Prostaglandin I_2 may act as a circulating hormone. For these reasons a series of experiments were performed to compare the maternal and fetal adrenal responses to prostaglandin I_2.

Materials and methods. Surgery was performed on 13 pregnant ewes from 126–129 days of gestation. The sheep were anesthetized with 10 mg/kg Nembutal (Abbott Laboratories) via a jugular catheter and given a spinal anesthetic of 100 mg Xylocaine in 7.5% glucose (Astra). Polyvinyl catheters (1.2 mm o.d., 0.7 mm i.d.) were placed into branches of both maternal femoral arteries and advanced 20 cm. A polyethylene catheter (PE 205), with a polyvinyl catheter inside, was inserted into the common carotid artery and advanced into the left ventricle. The catheter position was confirmed by the characteristic pressure pattern. The abdomen was then opened through a midline incision and the uterus exposed. A fetal hindlimb was brought through an incision in the uterine wall and the ventral surface was anesthetized with subcutaneous Xylocaine. Catheters were placed in the fetal hindlimb artery and vein using polyvinyl catheters similar to those used in the mother. These were advanced approximately 20 cm. The fetal leg incision was closed and another polyvinyl catheter was attached to the surface for measuring intrauterine pressure. The uterus was then closed. All catheters were filled with heparin (100 units/ml) and secured in a pouch on the maternal flank. The maternal jugular and carotid catheters were secured to the neck with an elastic bandage.

Two days after surgery the animal was returned to the laboratory and allowed to stabilize for at least 2 hr before the experiments began. At this point maternal and fetal blood pressures were stable. Maternal and fetal arterial blood samples were taken for blood gas analysis to describe the physiological state of the preparation.

Experimental protocol. Regional maternal and fetal blood flow measurements were made using the microsphere technique as previously described (7, 8). A control injection of radioactive microspheres was given either in the maternal left ventricle or fetal hindlimb vein while withdrawing an integrated arterial blood sample from either the maternal femoral or the fetal hindlimb artery. A bolus injection of prostaglandin I_2 was then given into the maternal left ventricle (1.0 mg) or into the fetal hindlimb vein (0.5 mg). The second microsphere injection was then given 90 sec later at the time of maximum response as indicated by the drop in blood pressure.

The radioactive microspheres were 25 μm in diameter and were labeled with ^{125}I, ^{85}Sr, ^{46}Sc (3M Company), ^{57}Co, or ^{113}Sn (New England Nuclear). The injectate contained approximately 1,000,000 (maternal) or 500,000 (fetal) microspheres labeled with a randomly selected isotope.

The prostaglandin I_2 (Upjohn Company) was stored in a freezer at −20° in vials each containing 5 mg. On the day of the experiment a vial was diluted with 1 ml of ethanol and 4 ml of saline to a concentration of 1 mg/ml, held on ice, and used within 2 hr.

At the completion of the experiment the

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as sacrificed with an overdose of Nem- 50 mg/ml) followed by an intracardiac on of saturated KCl. The appropriate were removed, weighed, and placed in ınd counted in a Nuclear Chicago 3 el gamma counter (Model 1185) with ɔpropriate integrated blood samples. flows were calculated from the gamma :r output on an interactive computer :tion with a Univac 1110 computer on ıs. Maternal arterial blood pressure was ɔred throughout the experiment. Ma- vascular resistance was defined as the arterial blood pressure divided by the flow to the organ in question. Fetal pressures were taken before and after ons and the average used to calculate nce. Fetal vascular resistance was de- ıs the mean arterial minus venous pres- ivided by the blood flow to the organ :stion. Comparisons were made using udent's paired or unpaired *t* test as ɔriate and the results were expressed as ± SE of the mean.

ılts. The maternal adrenal responses ıown in Table I. Blood flow to the ıal adrenal did not change significantly ırostaglandin I_2. However, there was a se in the blood pressure from 103.0 ± 64.0 ± 5.7 mm Hg ($P < 0.001$) with an panying decrease in adrenal vascular nce from 72.00 ± 6.65 to 52.60 ± 6.62 mm Hg/(ml/min)/g ($P < 0.001$).

The fetal adrenal responses are shown in Table II. There was a significant increase in blood flow per gram of tissue from 3.36 ± 0.22 to 6.64 ± 0.88 ml/min ($P < 0.01$). The fetal blood pressure decreased from 38.5 ± 1.2 to 28.3 ± 1.8 mm Hg ($P < 0.001$) and the adrenal vascular resistance decreased from 11.76 ± 0.98 to 6.13 ± 1.6 mm Hg/(ml/min)/g ($P < 0.01$). The fetal adrenals had a significantly higher flow per gram of tissue than the maternal adrenals in both the control and test conditions ($P < 0.001$).

The average number of spheres in the fetal adrenals was 887 ± 172 and 1435 ± 197 for the control and test injection, respectively. The fetal integrated blood samples contained 1632 ± 406 spheres and 1273 ± 256 spheres for the control and test conditions, respectively. The number of spheres in the maternal adrenals was 721 ± 105 in the control condition and 533 ± 142 in the test condition. The intergrated control blood samples had 625 ± 209 spheres and 464 ± 131 spheres in the test condition. The maternal arterial blood had a mean pH of 7.56 ± 0.01, pO_2 of 68.8 ± 3.2, and pCO_2 of 22.8 ± 1.0. Blood drawn from the catheter in the fetal hindlimb vein had a pH of 7.39 ± 0.01, pO_2 of 18.5 ± 1.1, and pCO_2 of 35.5 ± 3.9. Average fetal weight was 2.93 ± 0.29 kg.

Discussion. The use of radioactive micro-

TABLE I. MATERNAL ADRENAL CARDIOVASCULAR RESPONSES TO 1.0 MG PROSTAGLANDIN I_2

No.	Adrenal weight (g)	Blood flow (ml/min × g)		Resistance mm Hg/(ml/min)/g		Blood pressure (mm Hg)	
		Control	Test	Control	Test	Control	Test
	3.227[a]	1.71	1.63	73.10	49.08	125	80
	4.291	2.05	2.28	60.98	35.09		
	1.890[a]	2.02	1.49	46.54	29.53	94	44
	2.178	2.11	2.05	44.51	21.46		
	5.052[a]	1.23	0.96	67.48	87.50	83	84
	4.610	1.31	1.33	63.36	63.16		
	4.536[a]	0.92	0.51	100.00	94.12	92	48
	4.030	1.05	0.51	87.62	94.14		
	2.442[a]	2.00	2.00	55.00	30.50	110	61
	1.930	1.72	1.45	63.95	42.07		
	3.174[a]	0.86	0.90	122.09	66.67	105	60
	2.004	0.91	1.16	115.39	51.72		
	3.597[a]	2.07	2.14	54.11	32.71	112	70
	3.839	2.08	1.81	53.85	38.67		
ın	3.343	1.57	1.44	72.00	52.60	103	64
1	± 0.294	± 0.13	± 0.16	± 6.65	± 6.62	± 5.4	± 5.7
		$P < .295$		$P < 0.001$		$P < 0.001$	

icates left adrenal.

TABLE II. FETAL ADRENAL CARIOVASCULAR RESPONSES TO 0.5 MG PROSTAGLANDIN I_2

Sheep No.	Adrenal weight (g)	Blood flow (ml/min × g)		Resistance mm Hg/(ml/min)/g		Blood pressure (mm Hg)	
		Control	Test	Control	Test	Control	Test
8	0.438	3.998	7.717	8.75	2.85	35	22
9	0.315	3.965	2.790	10.34	11.83	41	33
10	0.367	2.651	9.074	15.09	3.42	40	31
11	0.191	3.351	6.675	10.74	4.35	36	29
12	0.286	2.986	7.531	14.07	10.38	42	31
13	0.246	3.201	6.075	11.56	3.95	37	24
Mean	0.307	3.359	6.644	11.76	6.13	39	28
SEM	± 0.036	± 0.219	± 0.876	± 0.98	± 1.60	± 1.2	± 1.8
		$P < 0.01$		$P < 0.01$		$P < 0.001$	

spheres to measure regional blood flow is a well-established technique. Buckberg *et al.* (9) have stated that a minimum of 400 spheres should be contained in each sample and this criterion has been met in our study. Subdiaphragmatic blood flows in the fetus can be measured with microspheres using the fetal hindlimb artery for the integrated blood sample and the hindlimb vein for the microsphere injections.

Due to our inability to determine fetal weight prior to the experiment and the need to elicit a sizable pressure response, a higher dose of prostaglandin I_2 was given to the fetus. A dose of prostaglandin I_2 was selected which produced approximately the same proportional blood pressure change in the mother and fetus. These values were −37.8 ± 5.1% and −26.5 ± 3.2%, respectively. The higher dosages also ensured the active prostaglandin I_2 was indeed getting to the organ in question, as prostaglandin I_2 is known to have a short half-life. Therefore the magnitude of the responses of the maternal and fetal adrenals to prostaglandin I_2 in these experiments cannot be compared.

The maternal adrenal control blood flow per gram of tissue seen in our study, 1.57 ± 0.13 ml/min×g, is similar to the value of 1.36 ± 0.20 ml/min×g reported by Rosenfeld *et al.* (10).

Tripp *et al.* (3) have reported a twofold increase in fetal adrenal blood flow with prostaglandin E_1 infusion (0.5–5.0 μg/min) in sheep. We observed a sinilar response to a bolus injection of prostaglandin I_2 in our experiments at a much higher dose (0.5 mg). They reported control flows of 0.8 ± 0.2 mg/min×kg fetus which are higher than ours, 0.40 ± 0.06 ml/min×kg fetus. It would appear that both prostaglandin E_1 and I_2 cause vasodilatation in the vasculature of the fetal adrenal gland.

Prostaglandins may be important in steroidogenesis. Dazord *et al.* (11) have shown that prostaglandin receptors exist in the ovine adrenal glands. An increase in corticosteroidogenesis after prostaglandin administration has been reported in the rat (5, 12), beef adrenal slices (13), and in the sheep fetal adrenals (14). In a study with chronically prepared dogs it was shown that increases in adrenal blood flow elicit increased cortisol secretion (15). Our results suggest that these responses may be associated with prostaglandin-induced hyperemia.

Summary. Fetal (n = 6) and maternal (n = 7) adrenal blood flows were measured with radioactive microspheres in chronically catheterized sheep, before and 90 sec after the injection of prostaglandin I_2. Adrenal vascular resistance is defined as the mean arterial pressure (maternal) or arteriovenous pressure difference (fetal) divided by the adrenal blood flow. Both the mother and the fetus responded with a decrease in blood pressure from 103 ± 5.4 to 64 ± 5.7 mm Hg and 39.0 ± 1.2 to 28.0 ± 1.8 mm Hg, respectively. Adrenal vascular resistance per gram also decreased in the mother from 72.00 ± 6.65 to 52.60 ± 6.62 mm Hg/(ml/min)/g and in the fetus from 11.8 ± 1.0 to 6.1 ± 1.6 mm Hg/(ml/min)/g. Prostaglandin I_2 did not significantly change the flow per gram to the maternal adrenals. The fetal adrenal flow per gram doubled in response to prostaglandin I_2. As the doses of prostaglandin I_2 are dissimilar the magnitude of the changes cannot be

compared. However, prostaglandin I_2 caused vasodilatation in both the maternal and fetal adrenals. Fetal adrenal control blood flows were significantly ($P < 0.001$) greater than the maternal control adrenal blood flows.

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Follow-Up Surveillance for Antibody in Human Subjects following Live Attenuated Measles, Mumps, and Rubella Virus Vaccines (40675)

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The value of live attenuated measles, mumps, and rubella virus vaccines is necessarily dependent on the continued presence of circulating antibodies which, in turn, are the basis for immunity. The present report describes the findings in follow-up studies to measure the levels of antibody following vaccination with monovalent live virus vaccines: 10 years after measles vaccines (Enders' Edmonston, Moraten line (Attenuvax), and Schwarz line); nearly 12 years after Jeryl Lynn strain mumps vaccine (Mumpsvax); and 9 and 9.5 years after duck cell modified HPV-77 rubella vaccine (Meruvax) in adult women and children, respectively. These vaccines were licensed for general use in the USA between 1963 and 1969: Enders' original (Edmonston) measles 1963; Moraten line measles (Attenuvax) 1968; Jeryl Lynn strain mumps vaccine (Mumpsvax) 1967; and HPV-77 duck rubella vaccine (Meruvax) 1969.

Materials and methods. Previous reports (1–18) gave detailed descriptions of the vaccines and the clinical trials in which they were investigated. The methods for surveillance were also presented (1, 3, 5, 7). The late bleedings required to carry out the present investigations were obtained with informed written consent and in compliance with the Investigative New Drug laws. The serum samples were tested using procedures described previously (1–18). In the tests, the last previous serum sample was reassayed in the same test with the new serum sample employing the same test procedure throughout. The close agreements in the values obtained provided assurance that differences in serum titer were not being introduced by the test itself. The parents, guardians, or older children were queried at the time of the late bleedings as to whether they had been revaccinated or had experienced the illness in the intervening period following vaccination. None of the children was revaccinated and none developed the corresponding illness since the time of inoculation.

Results. Measles virus vaccine. During May and June, 1967 (3), 256, 273, and 248 children who resided in suburban Philadelphia and who were seronegative for measles were given, respectively, Enders' original Edmonston measles vaccine or the Moraten or Schwarz lines of more attenuated measles virus vaccine. A portion of the children were followed at 1- to 2-year intervals to determine the titers of their measles hemagglutination-inhibiting (HI) antibodies. The parents and children reported no natural measles and no administration of additional measles virus vaccine. The serologic findings in the tests performed on serum samples taken 1 month and at 10 years after vaccination are given in Fig. 1. Decreases and increases in antibody were evident. Importantly, the antibody level of only one person fell to less than detectable level (Enders' Edmonston). Only the Enders' Edmonston group showed significant decline ($P < 0.01$) in mean antibody titer (63%) during the 10 years following vaccination at which time it was at a level similar to that of the Moraten and Schwarz line vaccine recipients. The least decline in mean antibody titer (24%) was seen with the Moraten line vaccine (Attenuvax), (cf. Schwarz, 31%).

Mumps virus vaccine. A controlled field study of mumps virus vaccine was initiated in the fall of 1965 in family and school populations in the Havertown–Springfield suburb of Philadelphia (4–6). Twenty-two children who were vaccinated in the study, as well as 24 unvaccinated control children in whom natural mumps developed shortly after the time that the vaccine was given, were bled periodically for up to 141 months after vac-

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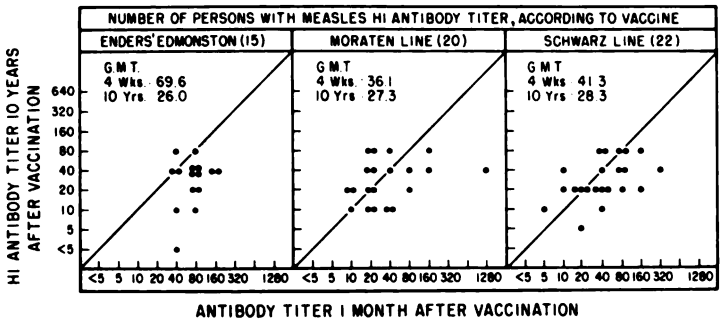


FIG. 1. Retention of measles HI antibody 10 years after vaccination with Enders' Edmonston, Moraten Line, or Schwarz Line measles vaccine (Study 97).

cination or onset of natural mumps and the serum samples were titrated for mumps neutralizing antibody. There was no readministration of mumps vaccine and no reported occurrence of natural mumps in the vaccinated persons. The antibody titers are given in Fig. 2. The initial level of neutralizing antibody reached after vaccination was usually considerably less than that following natural infection. However, a mumps neutralizing antibody titer of 1:1 or greater, in all but rare instances, conferred solid immunity against natural mumps (6). The levels of antibody among persons who had been vaccinated were just as great as among those whose antibody was the result of infection in nature. The mean titers following vaccination fell only 27% in nearly 12 years while those following the natural infection fell 80% during the approximately same time period to nearly the same level (mean 11.5) as following vaccination (mean 9.1). Subclinical natural reinfection might have occurred more frequently in the vaccinees than in those whose first experience with the virus had resulted from infection in nature.

Rubella virus vaccine. Children. The first large-scale clinical studies of HPV-77 duck cell modified rubella virus vaccine were initated in the Havertown–Springfield suburb of Philadelphia during September 1967 (7, 8). Fifty initially seronegative children who were vaccinated in 1967 were selected at random and bled 5.5, 7.5, and 9.5 years after vaccination. The serum samples were tested for rubella HI antibody and the results are shown in Fig. 3. The parents and children reported no natural disease and no administration of additional rubella vaccine. There was an expected initial decline in mean antibody titer at 2.5 years (mean 34.5) (18) with only slight reduction thereafter. One vaccinee was without detectable rubella antibody 9.5 years after vaccination. One of the two vaccinees who were without detectable rubella HI antibody (titer <1:8), after 7.5 years demonstrated an antibody titer of 1:8 9.5 years following vaccination. This difference might have been due to subclinical reinfection in nature during the intervening time period.

Adults. Sixteen initially seronegative adult women who were given HPV-77 rubella vaccine in 1968 (9) were bled again 5 and 9 years later and their sera were tested for rubella HI antibody. Fig. 4 shows that there were both decreases and increases in antibody. There was a greater than twofold increase in mean HI antibody titer at 5 years (94.5 versus 43.3) and this fell only slightly by the end of 9 years. None of the women became seronegative. Thus, the antibody level was retained. Some of the subjects presented serologic evidence for subclinical reinfection in nature.

Discussion. Measles, mumps, and rubella vaccines have been in widespread, routine use for several years, principally as single-dose combined vaccines. Protective efficacy has been very high and the continued presence of antibody has been remarkable, resembling that occurring after natural infections. It was of interest that, while highest homologous antibody levels were achieved following less attenuated measles vaccine (Enders' Edmonston) and following natural mumps compared with Moraten measles and mumps vaccine, respectively, this differential was lost with time. The mean titers 10 years after vaccination with measles vaccine were nearly

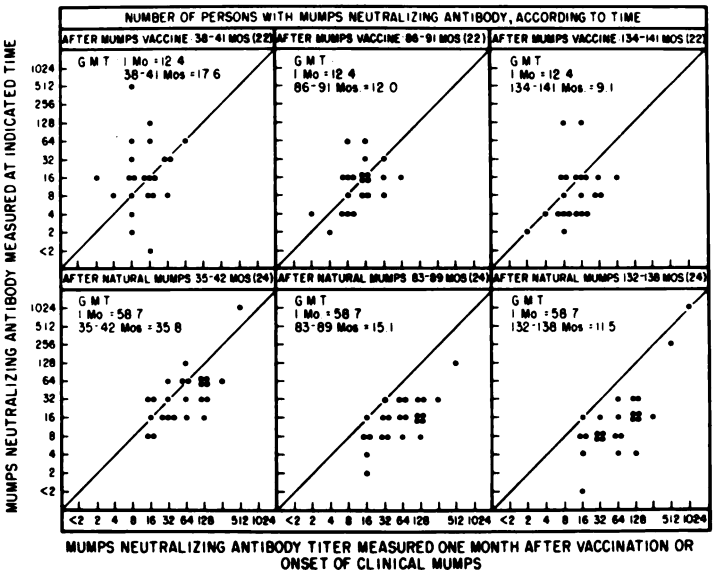

NUMBER OF PERSONS WITH MUMPS NEUTRALIZING ANTIBODY, ACCORDING TO TIME
AFTER MUMPS VACCINE 38-41 MOS (22)
AFTER MUMPS VACCINE 86-91 MOS (22)
AFTER MUMPS VACCINE 134-141 MOS (22)
AFTER NATURAL MUMPS 35-42 MOS (24)
AFTER NATURAL MUMPS 83-89 MOS (24)
AFTER NATURAL MUMPS 132-138 MOS (24)
MUMPS NEUTRALIZING ANTIBODY MEASURED AT INDICATED TIME
MUMPS NEUTRALIZING ANTIBODY TITER MEASURED ONE MONTH AFTER VACCINATION OR ONSET OF CLINICAL MUMPS

FIG. 2. Duration of neutralizing antibody following Jeryl Lynn strain live attenuated mumps virus vaccine compared with that following natural mumps (Study 71).

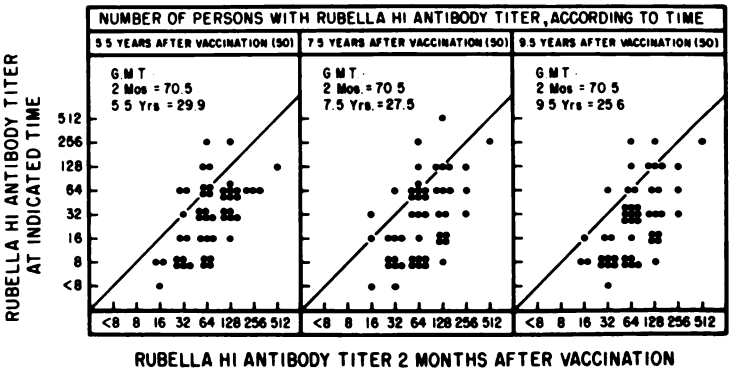
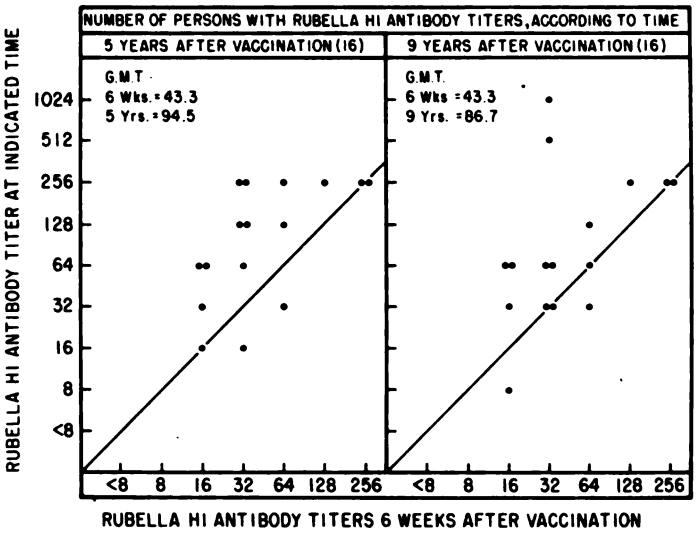

NUMBER OF PERSONS WITH RUBELLA HI ANTIBODY TITER, ACCORDING TO TIME
5.5 YEARS AFTER VACCINATION (90)
7.5 YEARS AFTER VACCINATION (90)
9.5 YEARS AFTER VACCINATION (90)
RUBELLA HI ANTIBODY TITER AT INDICATED TIME
RUBELLA HI ANTIBODY TITER 2 MONTHS AFTER VACCINATION

FIG. 3. Persistence of rubella HI antibody in children after administration of HPV-77 duck rubella vaccine (Study 153).

the same following all three measles vaccines (Enders' Edmonston, Moraten (Attenuvax), and Schwarz) and the mean titers nearly 12 years after natural mumps or Jeryl Lynn mumps virus vaccine were nearly the same. It may be that immunity following vaccination was more permissive of subclinical reinfection in nature than after natural disease with consequent boost in titer. Whatever the reason, it was of some importance that there was no apparent advantage, immunologically, of natural infection compared with vaccination.

The occurrence, in recent years, of clinical measles among persons who were vaccinated (19–26) has been explained on several bases: (a) use of killed virus vaccine that affords only short-term immunity, (b) the usual and expected failure of serologic response in 2 to 5% of persons who were given the vaccine under proper conditions, (c) the use of live measles vaccine of low or no potency owing to improper handling, storage temperature, overexposure to light, etc., (d) administration of the vaccine concomitantly with human immune globulin as originally carried out routinely with Enders' original vaccine or was improperly done with the more attenuated



3. 4. Persistence of rubella HI antibody in adult women after administration of HPV-77 duck rubella vaccine y 169).

sles virus vaccines, and (e) neutralization ve virus in the vaccine by maternal mea- antibody in infants less than 15 months ge. Validity for the conclusions relating educed takes when the vaccine is given r 12 months, but before 15 months, of age irrently disputed (27).

Whatever minor deficiencies may have n found, live attenuated measles, mumps, rubella virus vaccines have performed ptionally well (27–30) and continue to ear likely to provide lifelong protection nst clinical disease. The findings in these ies of excellent persistence of antibodies nst rubella virus following HPV-77 duck vaccine given in children and adult nen 9.5 or 9 years earlier are in accord n the findings recorded earlier by Meyer l. (31) and by Hermann *et al.* (32). They at variance with the declines in antibody rted by Horstmann (33) and by Balfour l. (34) in studies with the same vaccine.

ummary. Hemagglutination-inhibiting bodies were present at comparable levels ears after vaccination with Enders' orig- Edmonston and more attenuated Mora- (Attenuvax) and Schwarz line measles ines. There was no substantial decline in ralizing antibody titer for nearly 12 years administration of Jeryl Lynn strain mps virus vaccine (Mumpsvax). Hemag- nation-inhibiting antibodies were present without important decline in amount for at least 9.5 and 9 years, respectively, in children and adult women after administration of HPV-77 duck cell modified rubella vaccine (Meruvax). Higher homologous antibody titers were achieved initially following less attenuated Enders' Edmonston measles vaccine compared with more attenuated Moraten line vaccine and following natural mumps compared with mumps vaccine. Approximately 10 years later, however, the mean titers were roughly the same in each instance, indicating no apparent advantage of more virulent vaccine virus or natural infection in providing immunity. In our opinion, the relatively small number of purported measles virus vaccination failures reported in recent years were due mostly to mishandling or misadministration of the vaccine and not to deficiencies in measles vaccine itself.

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Nicotine Reduces Embryo Growth, Delays Implantation, and Retards Parturition in Rats (40676)

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Cigarette smoking has been implicated in a number of reproductive disorders in women (1, 2), and nicotine, a component of tobacco smoke, interferes with pregnancy in rats. Effects commonly reported are reduced litter size and weight and prolonged gestation (3–6). Studies in our laboratory demonstrate that administration of nicotine to pregnant rats modifies the process of implantation, delaying the attachment of blastocysts by as much as 9 hr (7). The present study was undertaken to determine if the nicotine-induced delay in implantation is associated with altered embryo development. Therefore, the time of zona pellucida loss, growth of the inner cell mass and rate of cell proliferation were determined in embryos retrieved from control and nicotine-treated rats. The effects of the alkaloid on parturition and fecundity were also noted.

Materials and methods. Mature, virgin, female rats of Sprague–Dawley strain (Camm Research), 200–350 g body wt were maintained under environmental conditions controlled with respect to room temperature (20–25°) humidity and photoperiod (14-hr light, 10-hr dark, lights on at 6 AM). Animals were housed two per cage and provided free access to Purina Lab Chow and water. Vaginal smears were recorded daily and each rat completed at least two consecutive estrous cycles before use. Females were placed with fertile males on the evening of proestrus; the presence of sperm in the vagina during estrus denoted Day 0 postcoitum. Nicotine solution for injection (10 mg/ml saline) was prepared daily from concentrated nicotine (98%, Eastman Kodak). Animals were randomly assigned to control and experimental groups. Controls received saline injections; experimentals received subcutaneous injections of 5 mg nicotine/kg body wt twice daily (10:00 AM and 3:00 PM) on Days 0 through 5 postcoitum. At selected times each horn was flushed with 2.0 ml of physiological saline in order to retrieve those blastocysts either free within the uterine lumen or loosely attached to the uterine epithelium. This was accomplished by attaching the tubal end of each uterine cornu to a vertically oriented blunt-tipped hypodermic needle (18 gauge). A 70-g weight was attached to the syringe plunger to standardize the force and rate of flow through each uterine horn. A stopcock prevented premature flow until the horn was firmly secured to the needle. The flushing apparatus thereby provided a constant volume, force, and rate of flow of saline through the uterine lumen of each specimen examined so that variations in the number of blastocysts retrieved could be attributed exclusively to the tenacity of blastocyst attachment to the uterine wall (7). Embryos were counted and examined for the presence of a zona pellucida and blastocyst cavity. The cells of each embryo were then dispersed and their nuclei stained and counted (8).

The effects of nicotine on fecundity and time of parturition were determined in a second series of animals receiving either saline or twice-daily nicotine injections (Days 0–5), as above, and allowed to complete pregnancy. On Day 18, animals were placed in an apparatus that recorded the time of onset of parturition (9). Following delivery, young were counted, weighed, and sexed and the mortality rate was calculated. Differences between control and experimental means were assessed by Student's t test (two tailed, significance level $P < 0.05$).

Results. The effects of nicotine on implantation as indicated by embryo retrieval are shown in Table IA. Fertilized ova could first be flushed from uteri at 6 PM Day 3 of pregnancy in both control and nicotine-treated rats; the number retrieved did not differ significantly between control (1.2 ± 0.8) and treated (0.3 ± 0.3) groups. Thereafter the number of embryos retrieved increased progressively through 6 AM Day 4 and re-

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TABLE I. EFFECTS OF NICOTINE ADMINISTRATION ON IMPLANTATION AND EMBRYO DEVELOPMENT[a]

	Day 3		Day 4			Day 5		
	6 PM	9 PM	6 AM	Noon	9 PM	6 AM	Noon	6 PM	9 PM
A. Embryo retrieval rate: number of embryos/cornu									
Control	1.2± 0.8 (10)[b]	2.4± 0.8 (14)	6.5± 0.8 (8)	5.5± 0.5 (12)	5.1± 0.5 (18)	3.8± 0.8 (14)	0.1± 0.1 (36)	0	0
Nicotine	0.3± 0.3 (10)	1.1± 0.5 (16)	5.3± 0.5 (10)	5.2± 0.7 (12)	6.3± 0.8 (10)	2.6± 0.6 (24)	3.5± 0.5* (12)	3.9 ± 0.7 (12)	1.1 ± 0.5 (16)
B. Zona pellucida loss: % embryos with zona									
Control	100(10)	100(34)	100(52)	100(66)	9(92)	0(53)	0(3)		
Nicotine	100(9)	100(18)	100(52)	100(63)	83(75)	45(26)	0(85)		
C. Inner cell mass growth: % embryos with cavity									
Control	0(10)	0(34)	100(52)	100(66)	16(92)	6(53)	0(3)		
Nicotine	0(9)	0(18)	100(52)	100(63)	46(75)	43(26)	73(85)		0 (16)
D. Embryo cell proliferation: number of nuclei/embryo									
Control	12.6± 0.3 (10)	15.3± 1.5 (9)	33.6± 4.1 (10)	42.0± 3.2 (12)	53.0± 0.7 (8)	77.0± 1.4 (10)			
Nicotine	8.7± 0.3** (9)	10.0± 1.0* (10)	16.0± 1.0* (14)	22.2± 2.7* (10)	36.0± 1.5* (15)	60.0± 1.7* (9)			

[a] The s.c. injection of mg nicotine/kg body wt twice daily (10:00 AM and 3:00 PM) on Days 0 through 5 postcoitum.

[b] Mean ± SEM; significantly different from control at $^*P < 0.01$ or $^{**}P < 0.05$ ( ): A No. of cornua; B, C, and D No. of embryos.

mained constant at approximately six per horn through 9 PM Day 4 in both treated and control rats. Subsequently the number of retrievable blastocysts declined in both groups. The decrease in recovery continued in control rats until noon Day 5 when virtually all blastocysts had attained a stage of implantation sufficient to resist dislodgement. By contrast, an average of 3.9 ± 0.7 blastocysts/horn could still be recovered from treated rats at 6 PM Day 5, and 1.1 ± 0.5 blastocysts were retrievable as late as 9 PM Day 5.

All embryos of both control and treated rats were zona encased at noon Day 4 (Table IB). Whereas loss of the zona pellucida was rapid in controls, being completed in most embryos between noon and 9 PM, zona loss was markedly slower in embryos of nicotine-treated animals, the zona being retained by 83% of the embryos at 9 PM Day 4 and by 45% at 6 AM Day 5. Zona loss was not evidenced by all embryos retrieved from treated rats until noon Day 5, i.e., approximately 15 hr later than controls.

In the majority of control embryos, growth of the inner cell mass was sufficient to obliterate the blastocyst cavity by late Day 4, only 6% possessing cavities as late as 6 AM Day 5 (Table IC). As with controls, the number of retrievable blastocysts decreased with time in treated rats, but a higher proportion of them retained a cavity (46, 43, and 73% at 9 PM Day 4, 6 AM and noon Day 5, respectively).

The rate of cell proliferation was reduced in embryos of nicotine-treated rats (Table ID). Upon entering the uterus (6 PM Day 3), embryos of treated rats consisted of fewer cells (8.7 ± 0.3 vs 12.6 ± 0.3; $P < 0.05$). The mean number of cells/embryo remained less ($P < 0.01$) than that of controls at all subsequent times examined.

Control and nicotine-treated rats differed ($P < 0.001$) in mean time onset of delivery (Table II). In controls, parturition occurred from 7 AM to 5 PM Day 22 with a mean time of delivery of 9.8 ± 0.40 hr, whereas treated rats initiated delivery from 6 AM Day 22 to 6 AM Day 25 with a mean time of 17.3 ± 2.0 hr Day 22. Despite delayed delivery, litters of treated rats did not differ significantly from those of controls with respect to size, birth weight, sex ratio, or mortality (Table II).

TABLE II. TIME OF PARTURITION, LITTER SIZE, SEX DISTRIBUTION, MORTALITY, AND BIRTH WEIGHT OF NEONATES BORN TO CONTROL VS NICOTINE-TREATED RATS[a]

	Parturition				Sex		% Mortality		Mean weight (g)	
Group	No. rats	Onset: $\bar{X}$ time hr (Day 22)	No. rats	Litter size: pups/litter	% Male	% Female	Male	Female	Male	Female
Control	39	9.8 ± 0.4[b]	19	10.4 ± 2.4	43.9	56.1	13.7	4.5	6.1 ± 0.4	6.0 ± 0.4
Nicotine treated	34	17.3 ± 2.0*	18	8.9 ± 1.4	49.0	51.0	9.5	9.6	6.7 ± 0.4	6.5 ± 0.5

[a] The s.c. injection of 5 mg nicotine/kg body wt twice daily (10:00 AM and 3:00 PM) on Days 0 through 5 postcoitum.

[b] Mean ± SEM unless indicated otherwise; * significantly different from control at $P < 0.001$ (mean ± SD).

Discussion. The present study has established that administration of nicotine during the initial 5 days of pregnancy modifies: (i) the time of zona pellucida loss, (ii) the rate of embryonic cell proliferation, (iii) the time of implantation, and (iv) the time of onset of parturition. The fact that embryos of nicotine-treated rats possessed fewer cells than those of controls upon entering the uterus indicates that nicotine exerts a growth suppressing effect on conceptuses while they reside within the oviduct. Whether cells of the inner cell mass vs trophoblasts differ in susceptibility to the growth-retarding effects of nicotine remains to be established. However, marked differences in cell susceptibility to nicotine action were not readily apparent since growth of the inner cell mass was sufficient to obliterate the blastocyst cavity and support normal organogenesis, and trophoblast invasion was sufficient to result in functional implantation. Indeed, it is possible that, at the time of implantation, blastocysts of nicotine-treated rats do not differ from controls in cell number; they may merely take longer to acquire the number of cells consonant with nidation. On the other hand, perhaps trophoblast cells may be reduced in number without adversely influencing implantation.

This study extends previous observations regarding the effects of nicotine on implantation in the rat (7). Approximately half the embryos present in treated rats remain retrievable at noon Day 5. Such blastocysts continue to reside within the uterus up to 9 hr before becoming sufficiently attached to the uterine wall to resist dislodgement by the standard flushing procedure. Why only approximately 50% of the blastocysts are susceptible to the effects of nicotine remains to be determined. However, since ovulation is completed quickly and ovulation and insemination occur prior to initiation of treatment, it is unlikely that differences in vulnerability to nicotine-induced effects are related to differences in the age of ova and/or embryos. Since all embryos of treated rats entered the uterus at the same time as those of controls, differences in susceptibility to nicotine are not due to differences in duration of exposure to the oviductal and/or uterine environments. The percentage of blastocysts showing retarded growth approximated the percentage of either sex delivered; hence, whether the sex of the blastocyst is a determinant of susceptibility to nicotine action remains to be investigated.

While the factors regulating the duration of gestation in the rat are poorly understood, it is known that the time of parturition is influenced by photoperiod (9) and is related to litter size and fetal bulk (10). The timing of parturition in control rats was comparable to that previously reported (9). Although the cause of prolongation of gestation in nicotine-treated rats remains obscure, it may be related to the delay in implantation experienced by approximately half the blastocysts in rats receiving the alkaloid even though, relative to controls, no differences in litter size or fetal bulk were noted.

Nicotine may modify development by acting directly on the embryo since it is concentrated in uterine secretions (11) and taken up by blastocysts (12). In addition, the alkaloid may act indirectly to modify the environment in which the embryo develops by altering estrogen and/or progesterone secretion via its suppressive effects on luteinizing hormone (13) and/or prolactin (14) secretion. That nicotine reduces uterine competence to un-

dergo decidualization (15), a process dependent on a delicate balance of estrogen and progesterone (16), suggests that ovarian steroid secretion is modified. Similarly, while zona pellucida loss is dependent on trophoblast maturation (17), it is also influenced by estrogen-dependent conditions within the uterus (18). Thus, the effects of nicotine on zona loss may result from altered estrogen levels as well as from retarded trophoblast growth.

Nicotine is a potent vasoactive substance that may indirectly influence the embryo by altering the functional state of the reproductive tract. The normal development of the embryo is dependent on adequate supplies of oxygen and essential nutrients (19). Since, prior to implantation, the conceptus is unattached, all its metabolic needs are met by diffusion of substrates from the oviductal or uterine environment. The availability of oxygen (20) and other metabolic substrates is dependent on nutritive blood flow to the reproductive tract. Occlusion of the uterine blood supply (21) or administration of serotonin, a vasoactive substance, alters uterine–blastocyst interaction (22). Similarly, studies in progress indicate that the nicotine regimen employed in the present study induces a marked and protracted reduction in both oviductal and uterine blood flow that may result in alterations in intrauterine oxygen tension.

Injection of pharmacological doses of nicotine into female rats is clearly not equivalent to inhalation of the alkaloid by women smoking cigarettes (23). Differences in species, route of administration and dosage preclude extrapolating the results of the present study to problems of pregnancy related to tobacco use. However, since nicotine can modify embryo development without being embryotoxic or impairing fecundity, it may be useful for studying embryo–uterine interactions.

That a 31% reduction in cell number occurs in the embryos of nicotine-treated rats prior to their entry into the uterus indicates embryo vulnerability to agents administered to the mother during their sojourn through the oviduct. Among the provocative questions raised by this observation is whether nicotine exerts its growth-suppressing effects by acting directly on the embryo and/or indirectly by modifying the milieu interiéur of the reproductive tract.

Summary. Daily injections of nicotine during the initial 5 days of pregnancy reduces embryo growth, delays implantation, and retards the onset of parturition in rats. Nicotine administration does not alter litter size, birth weight, sex distribution, or mortality rates.

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Relationships between Ca^{2+}, Myosin Light Chains, and ATPase in Bovine Aortic Actomyosin: Presence of Ca^{2+}-Requiring Inactivation Factor (40677)[1]

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The important role of Ca^{2+} in regulating contractile activity in vascular smooth muscle is well recognized (1, 2). However, the mechanistic basis for this regulation has not been completely elucidated. Studies with contractile proteins from several different smooth muscles including avian gizzard (3–5), porcine stomach (6), and hamster vas deferens (7) suggest that the Ca^{2+} regulatory mechanism is linked to the thick myosin filament and involves Ca^{2+}-dependent phosphorylation of the 15,000- to 20,000-dalton myosin light chains. This contrasts with striated muscle where the regulatory mechanism is linked to the Ca^{2+}-receptive protein troponin located on the thin actin filament (8). Nevertheless, Mikawa *et al.* (9) claim that Ca^{2+} regulation in smooth muscle is independent of phosphorylation and is associated with an 80,000-dalton troponin-like moiety.

Recently, we reported that Ca^{2+}-dependent phosphorylation of the 15,500-dalton myosin light chains occurs in native actomyosin from bovine aorta (10). The K_m for Ca^{2+} (3×10^{-7} *M*) in this reaction was the same as the K_m for Ca^{2+} in activating aortic actomyosin ATPase. This suggests that the Ca^{2+} regulatory mechanism for actin–myosin interactions in vascular smooth muscle may also involve Ca^{2+}-dependent phosphorylation of the myosin light chains. The hypothesis is particularly attractive because myosin-linked regulation is present in vascular smooth muscle (11, 12).

Ca^{2+} regulation is rapidly lost in actomyosin from vascular smooth muscle (2). Whether or not this loss of Ca^{2+} sensitivity is associated with changes in the phosphorylatable light chains is unknown. In this context, it is important to recognize that in other tissues Ca^{2+} has also been shown to activate proteases which specifically degrade proteins of the contractile apparatus. A Ca^{2+}-stimulated protease which degrades α-actinin and the Z line of myofibrils has been described in skeletal muscle (13). In cardiac muscle, an apparently different Ca^{2+}-stimulated protease exists which degrades only the phosphorylatable light chain (14). It is therefore possible that actomyosin from vascular smooth muscle also contains a Ca^{2+}-dependent factor which degrades or inactivates proteins involved in regulating actin–myosin interactions.

In this study we have examined phosphorylation of aortic actomyosin in preparations which required Ca^{2+} for hydrolysis of ATP and in stored preparations which no longer required Ca^{2+} for hydrolysis of ATP. Emphasis was placed on determining (a) if the loss of Ca^{2+} regulation was associated with changes in the phosphorylatable light chains, and (b) whether or not the actomyosin contained a Ca^{2+}-stimulated factor which could inactivate the regulatory mechanism for actin–myosin interaction.

Materials and methods. The methods used for (a) preparation of bovine aortic actomyosin, (b) performance of sodium dodecyl sulfate (SDS) electrophoresis and isoelectric focusing, (c) determinations of actomyosin ATPase activity, and (d) detection of phosphorylation of the myosin light chains using [γ-^{32}P]ATP were described previously (10).

Superprecipitation of actomyosin was studied according to methods adapted from Ebashi (15). Briefly, a reaction mixture was prepared consisting of 18 m*M* morpholinopropanesulfonic acid pH 7.0 (MOPS), 10 m*M* $MgCl_2$, 10^{-5} *M* $CaCl_2$, 2 mg actomyosin/ml, and sufficient KCl to maintain ionic strength at 0.1. The mixture (2 ml) was transferred to a cuvette in a Gilford spectrophotometer which permitted continuous stirring of the cuvette contents and maintained temperature at 25°. The reaction was started by injecting

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10 μl of 200 m*M* Na_2ATP into the mixture so that its final concentration was 1 m*M*. Changes in absorbance were recorded at 660 nm. After 5 min of incubation 250 μl was withdrawn for SDS electrophoresis and isoelectric focusing. Similar mixtures containing 1 m*M* ethyleneglycol-bis(2-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA) in place of $CaCl_2$ were used to assess Ca^{2+} sensitivity of the preparation.

Temporal relationships between phosphorylation of the myosin light chains and hydrolysis of ATP by actomyosin ATPase were assessed using reaction mixtures containing 0.4 to 0.6 mg actomyosin/ml and 10 μCi [γ-^{32}P]ATP/ml (Amersham, specific activity 2.2 Ci/mmole). The total concentration of ATP in the reaction mixture was 1 m*M* so that its specific activity was 2.2×10^4 cpm/nmole. At appropriate intervals aliquots of the mixture were withdrawn for determination of inorganic phosphate (ATPase activity) and phosphorylation of the myosin light chains (10).

Unless otherwise indicated, all of the above experiments were performed within 24 hr of the isolation of native actomyosin. Preliminary experiments showed that storage of the preparations at 4° resulted in time-dependent loss of Ca^{2+} sensitivity. Progressive loss of Ca^{2+} sensitivity was determined by changes in ATPase activity in the presence and absence of Ca^{2+}, superprecipitation, and analysis of electrophoretic profiles. The same actomyosin preparations were also stored in the presence of 5 m*M* EGTA to test for the presence of a Ca^{2+}-dependent factor which contributed to the loss of Ca^{2+} requirement for activation of actomyosin ATPase.

Results and discussion. Twelve preparations of actomyosin were studied. The yield of actomyosin was 2.8 ± 0.3 mg protein per gram of aortic muscularis.

In freshly prepared actomyosin, the Mg^{2+}-stimulated hydrolysis of ATP was Ca^{2+} dependent in all 12 preparations. The specific ATPase activity of fresh preparations decreased by 90% when the Ca^{2+} in the reaction mixture was chelated with 1 m*M* EGTA (Table 1). In accord with earlier findings (10) isoelectric focusing (IEF) of reaction mixtures containing Ca^{2+} and ATP showed that phosphorylation of the myosin light chains occurred as evidenced by the progressive disappearance of the unphosphorylated light chain focusing at pH 5.05 and the appearance of a new phosphorylated light chain band focusing at pH 4.97. This change appears to specifically require ATP because it did not occur when ADP, AMP, cAMP, or sodium pyrophosphate was substituted for ATP (Fig. 1). We have previously shown that the change in IEF is Ca^{2+} dependent and associated with incorporation of ^{32}P from [γ-^{32}P]ATP into the new band at pH 4.97. The phosphorylation is limited to the 15,500-dalton myosin light chains (10). Our procedure has been adapted to detect phosphorylated light chains in intact vascular smooth muscle (16).

Results obtained during concomitant measurements of the time course for phosphorylation of the light chains and activation of the Mg^{2+}-stimulated actomyosin ATPase showed that light-chain phosphorylation preceded the release of inorganic phosphate (P_i) from

TABLE 1. INFLUENCE OF CA^{2+} AND EGTA ON Mg^{2+}-STIMULATED ACTOMYOSIN ATPase ACTIVITY OF Ca^{2+}-SENSITIVE AND Ca^{2+}-INSENSITIVE PREPARATIONS OF AORTIC ACTOMYOSIN

Preparation	Actomyosin ATPase activity[a] (nmoles P_i/mg/min)		Ca^{2+} Sensitivity (activity in 10^{-5} *M* Ca^{2+}/ activity in 10^{-5} *M* EGTA)	*P*[b]
	In 10^{-5} *M* Ca^{2+}	In 10^{-3} *M* EGTA		
Ca^{2+}-Sensitive	32.4 ± 2.6	2.6 ± 1.9	12.5	<0.001
Ca^{2+}-Insensitive	18.3 ± 2.1	15.4 ± 1.9	1.2	>0.1 (NS)[d]
P[c]	<0.005	<0.005		

[a] Values for ATPase specific activity are expressed as means ± 1 SE for determinations on 12 different preparations of actomyosin. Each preparation was assayed within 24 h of preparation (Ca^{2+}-sensitive) and again after storage for 5 to 7 days at 4°C (Ca^{2+}-insensitive).

[b] Shows *P* value for difference in specific activity measured in the presence of Ca^{2+} and in the presence of EGTA.

[c] Shows *P* value for difference in specific activity of Ca^{2+}-sensitive and Ca^{2+}-insensitive preparations.

[d] NS, Not significantly different.

n of the actomyosin ATPase (Fig. 2). ıple, 50% of the maximal amount of rylation (^{32}P incorporated) occurred y 30 sec of incubation at a time when no P_i release from ATP was yet Similarly, of the total amount of P_i during the entire 30-min period of , only 7% (60 nmol P_i) was detected min after the reaction was initiated. contrast, 92% of the ^{32}P incorporated new phosphorylated myosin light nd was found in the same interval. ıdings, obtained with preparations ımmalian vascular smooth muscle, reement with results obtained from zzard actomyosin (17). The results hat phosphorylation occurs prior to diated activation of the Mg^{2+}-stimyosin ATPase. We have calculated[2] about 15% of the phosphorylatable ins are actually phosphorylated unptimal conditions of Fig. 2 (i.e., after incubation). Because this is a native ;in preparation rather than a reconystem of purified proteins, it is probthere is a phosphatase activity presell as the kinase activity we are g. The value of 15% phosphorylaherefore, likely to represent a dyuilibrium between phosphorylation hosphorylation of the light chain. ally, it should also be noted that the rse for phosphorylation of myosin ins *in vitro* is compatible with the rse for the development of maximal force (2–4 min) in strips of vascular nuscle isolated from a variety of 18, 19) and the time course maximal triction in several different vascular *ivo* (20, 21).

he preparations became Ca^{2+} insen-

ılue for the phosporylation of the phosphorht chain was obtained as follows. Given a ivity of [γ-^{32}P]ATP = 2.2 × 10^4 cpm/nmol, ration of a maximum of 7 × 10^3 dpm into the (Fig. 2) represents 0.32 nmol of phosphate d. The reaction mixture contained about 1 :in of which at least 50% or 0.5 mg is myosin a molecular weight of about 2.6 × 10^5, this 1.1 nmol of myosin. Thus, assuming two ›f phosphorylatable light chain per myosin, e 2.2 nmol of light chain of which 0.32 nmol ıosphorylated.

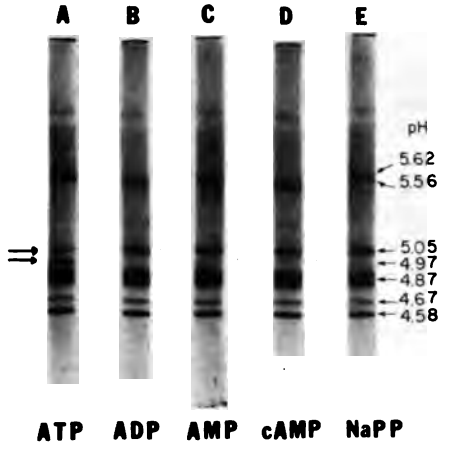

FIG. 1. Comparison of IEF of reaction mixtures containing equimolar (1 m*M*) concentrations of either ATP (A), ADP (B), AMP (C), cAMP (D), or sodium pyrophosphate (E). Each mixture contained 10^{-5} *M* $CaCl_2$. The gels were stained with Coomassie blue and each band can be located by the pH scale at right. Bands at pH 5.62–5.56 correspond to actin polymorphs, tropomyosin is in the diffuse area at pH 4.87, and the bands at pH 4.67 and 4.58 are unidentified. The unphosphorylated myosin light chain focuses at pH 5.05 (upper arrow at left), whereas the phosphorylated form focuses at pH 4.97 (lower arrow). The phosphorylated light chain (dotted line between gels) occurs only in the presence of ATP (A). The unphosphorylated band (solid line between gels) is intense in gels B–E.

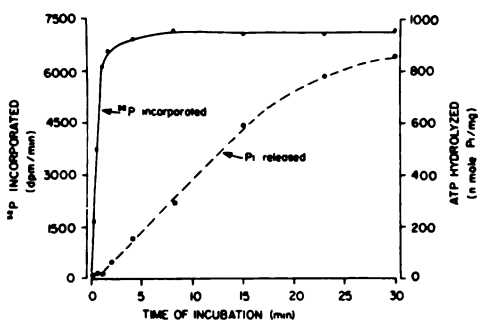

FIG. 2. Relationship between time of incubation at 25° (abscissa), incorporation of ^{32}P from [γ-^{32}P]ATP (left ordinate, solid curve), and release of inorganic phosphate (P_i) by Mg^{2+}-stimulated ATPase (right ordinate, broken curve). The incorporation of ^{32}P was determined by withdrawing aliquots from the reaction mixture at the times indicated and subjecting them to IEF and SDS electrophoresis. The myosin light-chain bands were excised and radioactivity was detected by scintillation counting (see Ref. (10)). Release of P_i was determined by ATPase assay. Phosphorylation was essentially completed before significant hydrolysis of ATP occurred by ATPase.

sitive after storage at 4° for 5 to 6 days so that the specific ATPase activity was similar in the presence and absence of EGTA (Table I). The loss of Ca^{2+} sensitivity was associated with the loss of the phosphorylatable myosin light chains (Fig. 3). It is particularly noteworthy that the loss of Ca^{2+} sensitivity also was associated with an increase in the basal Mg^{2+}-stimulated ATPase activity. Thus, while the specific ATPase activity of the aged actomyosin was decreased by 50% in the presence of Ca^{2+} it was increased, relative to the fresh actomyosin, by almost sixfold in the presence of EGTA (Table I). This marked increase in basal ATPase activity measured in the presence of EGTA may be related to the concomitant loss of Ca^{2+} sensitivity and of the phosphorylatable light chains. Thus, it is possible that there are two effects of the Ca^{2+}-dependent phosphorylation of light chains. First, phosphorylation eliminates the inhibition of actin-myosin interaction imposed by the light chain in its nonphosphorylated form. Second, the presence of the phosphorylated light chain augments actin activation of myosin ATPase. Accordingly, stored preparations which have lost the light chain would show both high basal ATPase activity and little or no stimulation with Ca^{2+}. Although additional studies are required to test this hypothesis, it is interesting to note that removal of the phosphorylatable light chain from cardiac myosin enhances actin-myosin interaction (22). Interest in the cardiac findings is heightened because it is an actin-linked regulatory system.

The coincident loss of Ca^{2+} sensitivity and phosphorylatable light chains was prevented when actomyosin preparations were stored in the presence of 5 m*M* EGTA (Fig. 3). Ca^{2+}-Dependent superprecipitation of preparations stored in the absence of EGTA became progressively delayed and decreased in magnitude (Figs. 3A–D). At the same time, however, Ca^{2+}-independent superprecipitation became more pronounced. These changes in Ca^{2+} sensitivity were associated with progressive loss of the 15,500-dalton myosin light chains. In sharp contrast, preparations stored in the presence of EGTA over the same period of time retained the myosin light chains and the Ca^{2+} requirement for superprecipitation (Figs. 3E–F). Storage in EGTA invariably protected against time-dependent loss of Ca^{2+} sensitivity. Knowledge that addition of EGTA protects the biochemical integrity of aortic actomyosin preparations during storage should also prove useful to other investigators. The protective effect of EGTA is specific because no protection was afforded

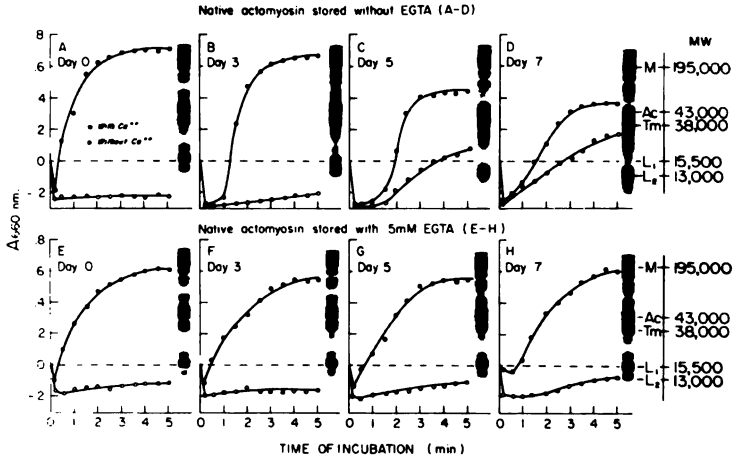

FIG. 3. Changes in Ca^{2+} sensitivity of actomyosin stored at 4° in the presence and absence of EGTA. Panels A-D show time-dependent changes in actomyosin stored for up to 7 days without EGTA. Superprecipitation was then measured turbidometrically in the presence of 10^{-5} *M* Ca^{2+} (●) or 10^{-3} *M* EGTA (○). SDS gels to the right of each panel show the changes in profiles with storage where M is myosin heavy chains, Ac is actin, Tm is tropomyosin, L_1 is the phosphorylatable light chain, and L_2 is the nonphosphorylatable light chain. Molecular weights are shown on scale at right. Panels E-F show corresponding material from the same actomyosin preparation stored in the presence of EGTA. Further details are given in the text.

when preparations were stored in the presence of soybean trypsin inhibitor, pepstatin, leupeptin, streptomyosin, chloremphenicol, sodium azide, or phenylmethylsulfonyl fluoride. The findings suggest that the aortic native actomyosin contains a Ca^{2+}-dependent factor, perhaps a Ca^{2+}-dependent protease, which digests the phosphorylatable light chain and thereby promotes the progressive loss of Ca^{2+} sensitivity. To our knowledge this is the first report of the presence of a Ca^{2+}-dependent factor in preparations from mammalian vascular smooth muscle which has high specificity for degrading the myosin light chains. Despite its relative specificity for myosin light chains, the postulated Ca^{2+}-dependent protease(s) clearly also partially degrades certain other proteins as evidenced by the disappearance of several other gel bands (e.g., 55,000 daltons) and the appearance of new bands (e.g., 80,000 daltons). Consequently, the decrease in superprecipitability as well as the alterations in Ca^{2+}-sensitive ATPase activity could also be related to modification of these other proteins. In this context, it is noteworthy that tryptic digestion of gizzard actomyosin results in concomitant loss of phosphorylatable light chains and actin-activated ATPase (4). However, partial digestion of the same preparation with papain preserves Ca^{2+} sensitivity as long as the phosphorylatable light chain remains intact. Clearly, further studies are required to characterize the Ca^{2+}-dependent factor in our preparations and define its role in maintaining function in vascular smooth muscle.

Summary. Phosphorylation of the myosin light chains occurs in Ca^{2+}-sensitive aortic actomyosin. This phosporylation specifically requires ATP and is essentially completed before significant release of P_i by actomyosin ATPase can be detected. Progressive loss of Ca^{2+} sensitivity in preparations stored at 4° is associated with progressive loss of phosphorylatable light chains so that phosphorylation no longer occurs. Although Ca^{2+}-activated ATPase is depressed in these light chain-deficient preparations, basal ATPase measured in the absence of Ca^{2+} is increased. These data suggest that the unphosphorylated light chains inhibit actin-stimulated myosin ATPase, whereas phosphorylation of the light chains relieves the inhibition and amplifies actin stimulation of myosin ATPase. During storage there is a parallel loss of the Ca^{2+} sensitivity and phosporylatable light chains. Both losses are prevented when actomyosin is stored in the presence of EGTA. These findings suggest that aortic actomyosin contains a Ca^{2+}-dependent factor, perhaps a Ca^{2+}-stimulated protease, which is responsible for progressive loss of phosporylatable light chains. The results of the present study provide further support for the hypothesis that the Ca^{2+} regulatory mechanism for actin–myosin interactions in mammalian vascular smooth muscle involves Ca^{2+}-dependent phosphorylation of the myosin light chains.

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Prolactin Receptors in Organ Culture of Rabbit Mammary Gland: Effect of Cycloheximide and Prolactin (40678)

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The central role of prolactin in mammary gland physiology has been extensively studied both *in vivo* and *in vitro* (1) and prolactin receptors have been identified and purified in rabbit mammary gland (2–4). However, the essential steps involved in the mechanism of prolactin action, subsequent to prolactin binding to the plasma membrane, are unknown.

It is well established that the mammary gland can be maintained in organ culture and responds well to hormones (5, 6). In addition, mammary explants can be used as an experimental model to study the steps involved in the mechanisms of hormone action (7). The aim of this study was to verify the maintenance of prolactin receptors in mammary glands in organ culture, to assess the apparent turnover of receptors, and to describe the effect of large doses of prolactin on the levels of its own receptor.

Materials and methods. Organ culture. Pseudopregnancy was induced in New Zealand rabbits, 6 months of age, by mating with vasectomized males. Animals were killed by cervical dislocation at 12–14 days of pseudopregnancy. Mammary glands were removed and placed in a sterile petri dish. Under a laminar flow hood, 5- to 10-g portion of tissue was selected from the periphery of the gland, rinsed in medium 199 (Gibco), and placed in an Erlenmeyer flask containing 10 ml of medium 199. Approximately 500 mg of tissue was removed and dissected from fat and conjunctive tissue under a binocular microscope. Epithelial fragments were cut into small fragments of 1 to 2 mm^3. Explants were placed on stainless-steel grids (approximately 20 explants per grid) and five grids were placed in 10 × 35-mm petri dishes. Medium 199 supplemented with amino acids (four times the usual concentration of amino acids in the medium) and bicarbonate (2.2 g/liter) were added to the dishes to cover the grids taking care not to entirely cover the explants with medium. The petri dishes were placed in an incubator at 37° under a controlled atmosphere of 57% O_2, 5% CO_2, 38% N_2 (8). Explants were cultured up to 48 hr in the presence of insulin (5 μg/ml, Sigma), cycloheximide (1 μg/ml, Sigma), or ovine prolactin (5 μg/ml, NIH-P-S12, 35 IU/mg). At the end of the culture, the grids were placed on filter paper and the explants removed, weighed and frozen at −20°.

Membrane preparations. Approximately 500 mg tissue was homogenized in 4 ml of 0.3 *M* sucrose in a Turax homogenizer for 30 sec at medium speed and rehomogenized in a glass–teflon homogenizer. The homogenate was centrifuged at 12,000*g* for 15 min and the supernatant was recentrifuged at 105,000*g* for 90 min and the pellet resuspended in Tris buffer (25 m*M* Tris–HCl, pH 7.5, 10 m*M* $MgCl_2$) as described previously (9). Protein was determined by the Lowry procedure (10) using bovine serum albumin (BSA) as standard on membranes solubilized with 1 *N* NaOH.

Since prolactin does not appear to dissociate from its receptor during membrane preparation, for explants cultured in the presence of prolactin, it was necessary to desaturate prolactin receptors prior to their assay. This technique involves an *in vitro* incubation of crude membranes with 4 *M* $MgCl_2$ as has been described in detail for growth hormone (11) and prolactin receptors (12).

Receptor assay. Prolactin binding was assayed using 200 μg membrane protein incubated with approximately 100,000 cpm of ^{125}I-labeled human growth hormone (hGH, NIH, HS2160E, 2 IU/mg) iodinated using chloramine-T (13), in the absence and presence of 1 μg of ovine prolactin. Human growth hormone has been shown to have

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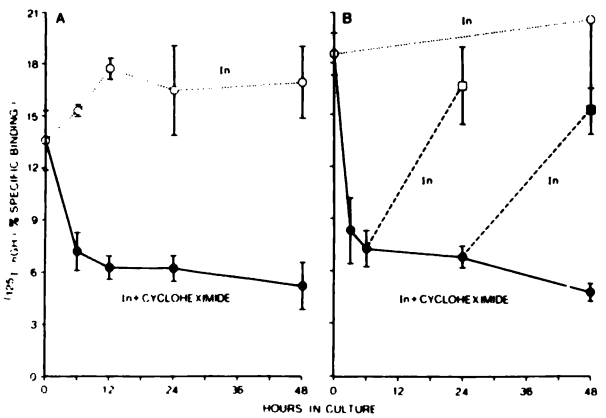
enic activity in the rabbit mammary (6) and has been used to identify pro- receptors in a number of target organs 'inal incubation volumes were adjusted ml with Tris buffer containing 0.1% Incubation proceeded for 16 hr at room rature and was stopped by the addition 1l of cold Tris buffer containing BSA. ıbes were centrifuged at 3500*g* for 15 .nd the pellets counted in a Packard .a counter.

ults. Figure 1A illustrates the mainte- of prolactin receptors in mammary explants cultured in the presence of ı. Binding increased slightly up to 12 hr :mained constant up to 48 hr. Addition lohexiмide (1 μg/ml) resulted in a rapid e of binding during the first 6 hr which ned low until 48 hr. Figure 1B shows er experiment and demonstrates the re- ility of the effect of cycloheximide (11). In the presence of insulin only, the)f receptors was maintained up until 48 shown previously. The addition of cy- :imide resulted in a rapid decrease of ors almost maximal at 3 hr. Removal lohexiмide from the culture medium at 6 or 24 hr by replacement with a .m deficient in cycloheximide resulted :turn of prolactin binding to near con- vels 18–24 hr later.

ıre 2 shows the effect of prolactin on /el of its own receptor in explants cul- in the presence of a concentration of prolactin classically used *in vitro* to induce secretory activity (1 to 5 μg/ml). Free and total receptors ($MgCl_2$-treated) were measured in explants cultured for 48 hr with insulin or insulin + prolactin. As expected, free receptors were approximately 80% saturated by 5 μg/ml prolactin. Surprisingly, total receptors were also markedly reduced from 12.34 ± 1.33 to 4.4 ± 0.78%, suggesting a down-regulation of prolactin receptor by prolactin. To exclude the possibility that $MgCl_2$ was not effective at this level of saturation, membranes from explants cultured in the presence of prolactin were completely saturated *in vitro* after which they were exposed to 4 *M* $MgCl_2$. The dissociation of prolactin was equally effective under these conditions, indicating that the loss of total receptors observed in Fig. 2 was not due to an artifact of the dissociation technique.

The time dependence of prolactin saturation and down-regulation of prolactin receptors in culture is shown in Fig. 3. Saturation was incomplete at 1 hr and was maximal at 24–48 hr. The pattern of the reduction of total prolactin receptors is different from that of free receptors with a maximal effect observed in explants cultured in the presence of prolactin for 48 hr.

Discussion. These studies demonstrate that mammary gland explants are capable of maintaining prolactin receptors up to 48 hr and that the maintainance is not a static phenomenon, but represents an equilibrium



1. Maintenance of prolactin receptors and the effect of cycloheximide on prolactin receptors in mammary xplants. (A) Mammary explants were cultured at different times in the presence of insulin (In) or In + ximide. (B) Mammary explants cultured in the presence of In or In + cycloheximide after which medium was l at 6 or 24 hr and the cycloheximide was removed. Values are means ± SEM of three cultures.

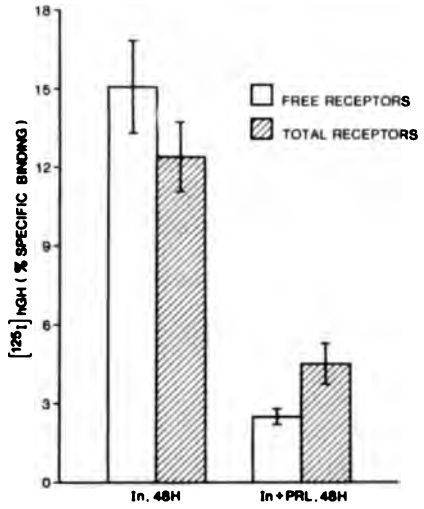


FIG. 2. The effect of prolactin (PRL) on the free and total level of its own receptor in organ culture of rabbit mammary gland. Explants were cultured in the presence of insulin (In, 5 μg/ml) or In + PRL (5 μg/ml) for 48 hr. Values are means ± SEM of seven or eight cultures.

between degradation and synthesis of receptors. Addition of cycloheximide to the explants revealed a marked loss of receptors, possibly indicating a rapid turnover. In rat liver, prolactin binding sites have been shown to be reduced by 90% within 2 hr after a single injection of 200 μg of cycloheximide with a return to normal levels 24–48 hr after injection (15).

In vitro, the massive doses of prolactin required to stimulate casein synthesis resulted in a 80% occupation of receptors. These receptors not saturated may represent sites inaccessible to the hormone. Magnesium chloride treatment of membranes from explants cultured in the presence of insulin alone in most cases led to a slight reduction of prolactin binding, most probably due to the loss of protein following treatment with the dissociating agent (12). For example, correction of total receptor levels shown in Fig. 2 for a 30% loss of protein results in 16.04 ± 1.73% binding, a value slightly higher than that observed for free receptors. In contrast, when explants were cultured with prolactin, treatment with $MgCl_2$ resulted in higher binding than in nontreated membranes. However, exposure to prolactin over a longer period of time (24–48 hr) led to a progressive reduction in binding corresponding to a relative reduction in total receptor levels. This down-regulation of prolactin receptors by prolactin itself is in contrast to the established stimulatory effect of prolactin on its receptor in mammary gland (16) and liver (17, 18) observed in chronically treated animals. We have observed a similar down-regulation of prolactin receptors *in vivo* in rabbit mammary gland and rat livers in animals which was maximal 6 hr after a single intravenous injection of prolactin (19). A decrease of prolactin binding following injection of increasing concentrations of prolactin has been reported in rat testis (20). This decline could however at last partially be explained by an occupation of free receptors, since total prolactin receptors (following dissociation) were not measured.

Down-regulation could reflect a compartmentalization of receptors which are progressively excluded from the crude membrane preparation we utilize. For other hormones such as LH (21) which induces a down-regulation of its own receptor, binding of the hormone to the plasma membrane is followed by an endocytosis of the hormone–receptor complex. In addition, injection of ^{125}I-labeled ovine prolactin into rats results in a rapid concentration of intact prolactin inside the cell within Golgi vesicles (22). This could occur in mammary gland also, especially in view of the fact that prolactin has been local-

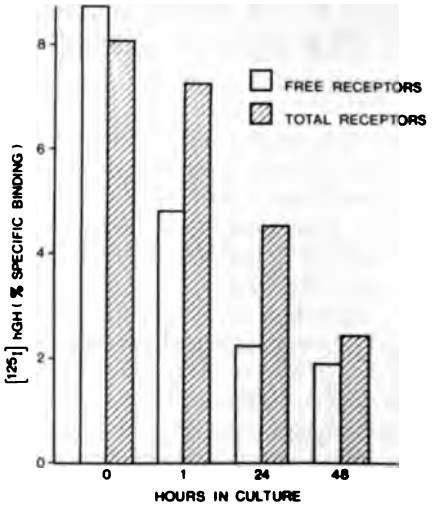


FIG. 3. Time course of the occupation of prolactin (PRL) receptors and decline in total ($MgCl_2$-treated) receptors in mammary explants cultured in the presence of insulin (In) and PRL (5 μg/ml) for the indicated times.

ized within mammary epithelial cells by immunohistochemical techniques (23). It is possible that down-regulation of prolactin receptors is more difficult to observe due to the apparent rapid turnover of receptors suggested *in vivo* for liver (15) and by the present studies *in vitro* for the mammary gland.

Summary. Prolactin receptors were maintained in organ cultures of rabbit mammary gland and this maintenance was related to an active synthesis since the addition of cycloheximide lead to a rapid decline in receptor levels. Inclusion of prolactin (5 μg/ml) in the culture medium resulted in an occupation of binding sites, followed by a reduction in the total receptor levels measured following dissociation of the bound prolactin from its receptor in crude membrane preparations. This apparent down-regulation of prolactin receptors may result from an endocytosis or compartmentalization of the hormone–receptor complex and may be involved in the mechanism of action of prolactin.

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Dietary Essential Fatty Acids Are Required for Maintenance and Induction of Prolactin Receptors (40679)

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There is a positive correlation between the incidence of mammary carcinoma and the dietary fat intake by the populations of various countries (1–4). The causal relationship suggested by these demographic observations has been supported by laboratory studies: animals on diets high in unsaturated fats are most likely to develop mammary carcinoma compared to the animals fed diets containing low concentrations of these fatty acids (5–9). Such diets which enhance tumor formation include the essential fatty acids (EFA), linoleic and linolenic acids. These dietary components have previously been shown to alter the fertility of rodents (10) and, therefore, possess the potential to modify certain hormone-related events. These observations plus the fact that some mammary tumors are stimulated to grow by high concentrations of prolactin (11) prompted us to hypothesize that essential fatty acids may alter hormonal effects by modulating the prolactin receptor present on the surface of target cells. Such modifications of the hormone–receptor axis could then alter the response of the cell to normal concentrations of hormones within the body and thereby provide a partial explanation of the role of diet in the development of mammary carcinoma.

Materials and methods. Essential fatty acid-deficient diet (No. TD 77052, Teklad Mills, Madison, Wisc.) was composed (w/w) of 68.5% sucrose, 20% casein, 5% medium chain triglycerides (MCT), 2% nonnutritive fiber, 3.5% mineral mix (Williams-Briggs Mod. No. 170911), and 1% vitamin mix (Teklad Mills No. 40060). MCT consist of resynthesized triglycerides from fatty acids of 8- to 14-carbon chain length hydrolyzed and subsequently purified from coconut oil. The control diet (No. TD 77053, Teklad Mills) was manufactured using the same recipe as above except the MCT was replaced by corn oil[1] and was, therefore, isocaloric with the deficient diet.

C_3H mice (Charles River) were fed EFA-deficient or control diets starting 1–2 weeks after being weaned at age 4 weeks from NIH lab chow-fed dams and were sacrificed by decapitation at various times during the subsequent 32 weeks. Some C_3H mice were also started on the diets in midpregnancy and continued on the same diets until the pups were weaned. These dams and their litters were then maintained on the same dietary regimens until sacrifice at various times thereafter. One group of weanling mice also received subcutaneous injections of 10 μl linoleic acid (Sigma) thrice weekly. Half of these animals were placed on the control diet and the other half on the deficient diet. They were sacrificed after 32 weeks on this regimen.

Ovine prolactin (oPRL, NIH-P-S12, 35 IU/mg) was a gift of the National Pituitary Agency, National Institute of Arthritis, Metabolism, and Digestive Diseases. Iodination of oPRL was performed using a modification of the Thorell and Johansson technique (12). Approximately 1 mCi ^{125}I (Amersham/Searle) was added to 5 μg unlabeled hormone and buffered with 10 μl 0.4 *M* Na-acetate, pH 5.6. One microliter of a lactoperoxidase solution (2.7 mg/ml, Calbiochem grade B enzyme) was added, followed by 1 μl H_2O_2 solution (30% diluted 1:15,000). Thirty seconds later, 100 μl of "transfer solution" (0.02% Na-azide, 1% KI, and 16% sucrose) was added to quench the reaction. The mixture was removed and the iodination vessel was rinsed with 100 μl transfer solution. These solutions were combined and chromatographed on a 1.5 × 90-cm Sephadex G-100 column at 4°, previously equilibrated with 0.025 *M* Tris, pH 7.6. Fractions high on

[1] Corn oil fatty acid profile, percentage by weight: lauric = 0.02, myristic = 0.13, palmitic = 10.8, palmitoleic = 0.38, heptadecanoic = 0.05, heptadecenoic = 0.03, stearic = 2.18; oleic = 23.0, linoleic = 39.8, linolenic = 0.92 (Raltech Scientific Services, Inc., Madison, Wisconsin 53707).

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the descending limb of the hormone peak were used and had an approximate specific activity of 60 μCi/μg.

Animals were sacrificed by decapitation at the times specified and trunk blood was collected. The excised livers were immediately placed in liquid nitrogen and pulverized. They were then homogenized (Polytron, Brinkmann) for 1 min at 4° in approximately 10 vol of 0.025 *M* Tris buffer, pH 7.6, containing 0.3 *M* sucrose. A preliminary 15,000*g* centrifugation at 4° for 20 min was performed and the supernatant was centrifuged at 100,000*g* for 60 min. These latter cell membrane pellets were stored at −40° for less than 4 days prior to use. Additional livers were obtained from mature female C_3H after receiving either control or deficient diets for 33 weeks and homogenized as above. After a preliminary 120*g* centrifugation at 4° for 20 min to remove nuclei and large particles, the supernatant was used as suspension of receptor for special study in one set of experiments.

Hormone binding activity was determined by measuring the amount of ^{125}I-labeled hormone specifically bound to liver membrane preparations under the following assay conditions: ~60,000 cpm of labeled hormone was incubated with the 100,000*g* liver membrane preparation (0.3–0.6 mg protein) and serial dilutions of unlabeled hormone (0–500 ng) in a final volume of 0.5 ml buffer (0.01 *M* $MgCl_2$ and 0.1% bovine serum albumin in 0.025 *M* Tris at pH 7.6). These preparations were incubated overnight at room temperature and the incubations were terminated by addition of 1 ml chilled buffer at 4°. The tubes were then centrifuged at 2500*g* for 20 min at 4°, and the pellets were rinsed with an additional 1 ml cold buffer and recentrifuged. The supernatant was decanted and the pellet counted in a Packard gamma counter. The counts per minute bound in the presence of 500 ng unlabeled hormone was used as the value of nonspecific binding was subtracted from the total counts in the remaining tubes in that assay group to yield binding values required for Scatchard analysis (13).

Serum samples or suspensions of 100,000*g* membrane pellets were extracted by shaking with 20 vol of chloroform:methanol (3:1) for 30 min at room temperature. The mixture was then centrifuged at 1500*g* for 10 min and the resulting organic phase passed through a glass filter prior to being evaporated to dryness under a nitrogen blanket. The samples were then incubated for 2 hr at 90° in 2 ml 30% KOH/absolute methanol, cooled to room temperature, and acidified to pH 1–2 with concentrated HCl. The free fatty acids were then extracted into 8 vol of hexane and evaporated to dryness with N_2. After solubilizing the samples in 1 ml methanol/phenolphthalein, they were neutralized with 0.1 *N* KOH/methanol, evaporated to dryness, and resolubilized in 1 ml of a uv tag solution composed of 15 mg α-*p*-dibromoacetophenone (Aldrich Chemical Co.) and 1.5 mg 18-Crown-6 reagent (Aldrich Chemical Co.) per 1 ml acetonitrile (Brudick and Jackson Laboratories). The solution was incubated for 30 min at 80° and cleared of particulate matter by centrifugation at 1500*g* for 10 min. Free fatty acids were separated by using high-pressure liquid chromatography (HPLC) techniques on a μBondapak C18 column with the Waters Associates Model 440 absorbance detector and solvent programmer 660 set to curve No. 5 eluting with 1 ml per minute at 40 to 85% acetonitrile over a 3-hr period (14, 15).

Results. The deficient animals gained weight slower and attained only two-thirds of the final weights of their control diet-fed littermates (Fig. 1) even though the average daily food consumption was the same for both groups. These animals exhibited the manifestations of EFA deficiency: lethargy, reddish fur coloring, rough coat, and dermatitis that developed after 22 weeks on the diet. The quantity of fat within both the peritoneal cavity and mammary fat pads was visibly decreased 2 months after starting the deficient diet at weaning and was virtually absent after 6 months.

Livers from both dietary groups were morphologically indistinguishable by light microscopy.

The 100,000*g* membrane pellets, which include plasma membrane and endoplasmic reticulum fractions, were prepared from the livers of these animals and assayed for prolactin binding (7).

Females, which normally have higher levels of liver prolactin receptors than males (16), showed their number of receptors to be

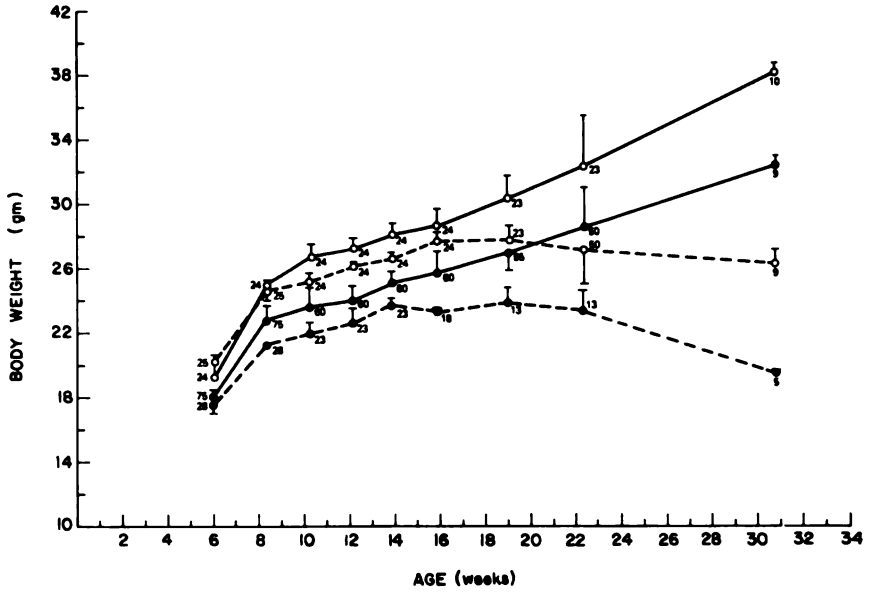

FIG. 1. Weight of C_3H male (O) and female (●) mice as a function of age and type of diet. Animals were weaned from mothers that had been fed standard NIH lab chow through pregnancy and nursing. The pups were continued on the standard diet until being placed on either the control (——) or deficient (- - -) diets 1-2 weeks after weaning. Vertical bars represent 1 SEM. Numbers represent the quantity of mice used to obtain each data point.

65 and 41% of controls after 22 and 32 weeks of being fed the deficient diet (Fig. 2). Scatchard analyses (13) revealed the apparent affinity constant, K_a, to be $10.5 \pm 0.6 \times 10^9$ M^{-1}, unchanged from the value of $9.8 \pm 0.6 \times 10^9$ M^{-1} for the control membranes. The changes observed, therefore, represent a decrease in the number of binding sites. Supplementation of these deficient animals by thrice weekly subcutaneous injections of 10 μl of linoleic acid (Sigma Chemical Co.) raised the number of receptors to 75% of controls after 32 weeks of dietary treatment. A similar pattern was observed at 16 weeks of age if the diet was started while *in utero*, ~1 week prepartum: deficient animals possess 59 ± 17% of the number of control prolactin receptors. Binding studies were also performed using crude tissue homogenate to determine if dietary-induced alterations in the sedimentation characteristics of the subcellular fractions were responsible for these observations. Pregnant dams maintained on their diets for 32 weeks after parturition had only 52 ± 12 and 46 ± 12% of the binding capacity of controls in the 100,000*g* membrane pellets and 120*g* membrane supernatants, respectively. These studies showed that

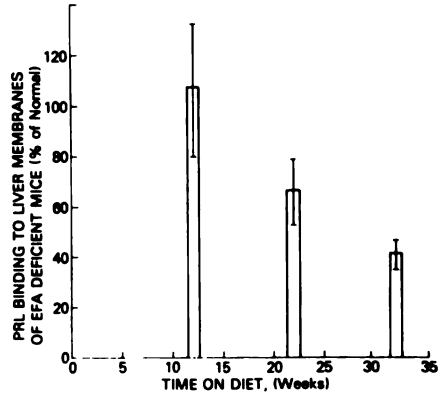

FIG. 2. Ovine PRL binding to 100,000*g* liver membrane pellets obtained from female C_3H mice fed essential fatty acid-deficient diets for the periods of time depicted on the abscissa. The ordinate represents the ratio of specific oPRL binding per milligram of protein observed in EFA-deficient diet-fed animals to that of control diet-fed animals. Bars represent standard errors. Scatchard analyses of these binding data demonstrate that the gradual decrease in binding represents a decrease in receptor number rather than a change in $K_a \approx 10 \times 10^9$ M^{-1}.

the EFA deficiency-induced decrease in oPRL binding was not due to an altered subcellular centrifugation profile but rather

to an actual change in the binding properties of the cell membranes.

Other investigators (18) have shown that rats eating diets containing 20% (w/w) corn oil have higher serum levels of PRL than rats eating diets containing 5% (w/w) corn oil. It has also been shown that PRL will induce its own receptor when injected ip into male mice (19). For these reasons, there was concern that the EFA deficiency was in some way lowering the serum levels of PRL and thereby suppressing the number of PRL receptors. This question was answered when 20 C_3H males receiving either the deficient or control diets for 22 weeks were injected with 50 μg bPRL or the saline carrier ip every 4 hr, for a total of 11 injections. The animals were sacrificed 8 hr after the last injection. The dietary controls responded with a four-fold increase in the number of prolactin receptors, whereas no increase was observed in deficient animals (Fig. 3). No suppression of PRL binding was noted in the saline-injected deficient mice when compared to saline-injected control diet mice. This indicated that even if prolactin levels had been suppressed by the EFA deficiency, the binding capacity could not be returned to normal even though the serum PRL had been returned to a level that would induce binding activity in the animal. This indicates that the primary change in the receptor is due to a modification of the membrane itself rather than a change in the concentration of hormone responsible for receptor binding activity. Assays by HPLC of lipid extracted from the hepatic microsomal fractions of the deficient animals showed linoleic and palmitoleic/arachidonic acids to be ~12% of control values. HPLC analyses of sera from animals fed the deficient diet for 12 weeks showed linoleic and arachidonic/palmitoleic acids to be 4 and 25% of the control values, respectively. These changes resulted in an increase in the fluorescence depolarization which reflects an increase in the viscosity of the 100,000g membrane pellets obtained from the livers of animals fed the EFA-deficient diets for 22 weeks when compared to the control diet-fed animals (20, 21).

Discussion. While the dietary-induced suppression of PRL binding was marked in the female, males showed no such percentage

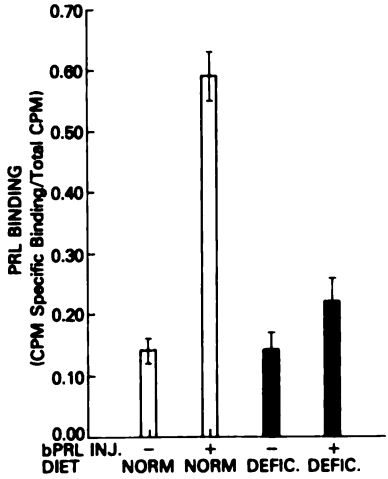

FIG. 3. Effect of an EFA-deficient diet on the ability to induce prolactin receptors. Ordinate is specific binding of oPRL (cpm) to 100,000g liver membrane pellet per milligram of protein divided by the total cpm added to each incubation tube (52,800 cpm). Abscissa depicts the *in vivo* treatment prior to sacrifice. Male C_3H mice were maintained on either EFA-deficient or control diets for 22 weeks and then injected ip with either 50 μg bPRL or Tris–saline carrier every 4 hr × 11 and sacrificed 8 hr after the last injection. Animals fed normal diets responded with fourfold increase in PRL receptor number when injected with bPRL while the EFA-deficient animals did not demonstrate a significant change in receptor numbers. Vertical bars represent standard error.

decrease. The reason for this is unclear but may be due to the fact that females are more susceptible to PRL-receptor modification because of either the inherently larger numbers of receptor sites in the basal states (17) or the susceptibility of the controlling hormonal factors to exogenous manipulation.

The PRL receptors present in very low levels in males may, however, be less susceptible than females to suppression by exogenous means; further suppression of what could be considered rudimentary levels may become increasingly more difficult as the number of sites decreases. The deficiency in dietary linoleic acid may exert these effects directly on the target tissue or may act indirectly via an intermediate tissue.

Receptors for certain hormones and antigens are considered to float within the lipid bilayer of the cell surface (22–25). Binding to receptors may, therefore, be modified not only by altering the structure of the binding site itself, but also by changing the character

of the matrix in which it exists. The latter may then affect the receptor configuration and/or its position relative to the extracellular, intramembranous, or intracellular environments (26). While the activity of several cell surface receptors has been modified *in vitro* by altering the lipids of either cell membranes or artificial vesicles (27–32), the data herein reported lend *in vivo* relevance to such observations. The essential fatty acids appear to be necessary not only for the maintenance of the hepatic prolactin receptor but also for its induction *in vivo*. The fact that induction did not occur when the deficient animals were treated with bPRL indicates that diet-induced suppression of endogenous prolactin (33) was not responsible but rather that another effect on the receptor–lipid complex was achieved. This may be a result of changes in the structural composition of the bilayer or the effect of decreasing the availability of these essential fatty acids for use as precursors of prostaglandins.

These data indicate that the response of tissues to lactogenic hormones can be modified by specific types of fat within the diet.

Summary. C_3H mice maintained on diets lacking the essential fatty acids exhibited a progressive decrease with time in the number of prolactin receptors detectable in their hepatic microsomal membranes. These deficient animals were also insensitive to induction of receptor by treatment with exogenous prolactin. These data show that the quality of dietary lipids modifies the prolactin receptor *in vivo*.

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Half-Life in Cirrhotics with High Secretory Volumes to a Secretin Test (40680)

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with hepatic cirrhosis frequently ncreatic and biliary hypersecretion e to exogenous secretin (1–3), but nism is unknown. One of the pro- lanations for this phenomenon is secretin stimulation resulting from catabolism by an abnormal liver for the liver in the catabolism of as been suggested by the work of , who demonstrated inactivation of ne by rat liver *in vitro* (5).

he purpose of this study to deter- ther or not patients with biopsy- rrhosis and secretin-induced pan- l biliary hypersecretion exhibit ab- cretin catabolism as reflected by an in its disappearance rate ($t_{\frac{1}{2}}$).

ls and methods. Four chronic alco- s (age 42–54) who had biopsy-doc- iepatic cirrhosis and were clinically cites were selected. All had been /aluated with routine liver function retention studies, creatinine clear- m amylase, and albumin (Table I). lies were compatible with a mild to degree of hepatic functional im- and normal renal function. All pa- erwent a standard secretin test of function using intravenous injec- unit of GIH secretin per kilogram ght. Each patient had a pancreatic volume greater than 4.9 ml/kg-hr ml/kg-hr) with normal values for itory being less than 4.6 ml/kg-hr. peak bicarbonate concentration an 80 meq per liter in two patients, rbonate output was normal in all, ter than 20 meq/hr (Table I).

unteers who were clinically free of enal, and pancreatic disease and ght approximated that of the cir- jects were selected as controls.

cretin at a dose of one unit per vas administered intravenously for istant infusion. Blood samples were efore the infusion, at 15-min intervals during the infusion, and at 2-min intervals for 20 min after completion of the infusion. Serum secretin concentrations were determined by a previously described sensitive and specific radioimmunoassay (6). Regression equations were determined from plateau infusion and postinfusion secretin levels after subtracting the basal (or preinfusion) values. The natural logarithm of the adjusted postinfusion values was used in linear regression analysis against time to determine the slope (K) of the regression line. The half-lives were calculated using the formula, $t_{1/2} = \ln \frac{1}{2}/-K$ (7).

The relationship of body weight to disappearance half-life was examined in each cirrhotic subject by linear regression analysis. In addition, the relationships of renal function (creatinine clearance), liver function (BSP retention), and pancreatic function (secretory volume, bicarbonate output, and bicarbonate concentration) to disappearance half-life in the cirrhotic subjects were also examined.

Results. Preinfusion and plateau mean secretin levels were similar in each group, being 53.2 and 678 pg/ml, respectively, for the cirrhotic group and 46 and 717 pg/ml, respectively, for the controls. In both groups plateau secretin levels were obtained within 30 min after starting the infusion and were maintained until the infusion was discontinued.

The cirrhotic group had a mean secretin half-life of 2.74 ± 0.31 (SEM) min (Fig. 1). The healthy volunteers showed a mean half-life of 2.72 ± 0.41 (SEM) min which was not significantly different from that of the cirrhotic patients ($P > 0.1$) (Table II).

Individual values for disappearance half-life were compared with parameters of hepatic, renal, and pancreatic function and weight by linear regression analysis. There was no correlation between secretin half-life and either BSP retention ($r = -0.09771$), creatinine clearance ($r = -0.45664$), peak bicarbonate concentration ($r = 0.76428$),

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TABLE I. LABORATORY DATA OF THE FOUR PATIENTS WITH CIRRHOSIS

Test	Mean	Range
Secretin test		
Volume	7.82 ml/kg-hr	(4.92–10.2)
Peak HCO_3–	77 meq/liter	(54–107)
HCO_3– output	43.5/meq-hr	(20.6–55.5)
Serum amylase	85 units	(35–134)
Liver function		
BSP (%)	14%	(4–34)
Serum albumin	3.7 g%	(3.1–4.3)
Prothrombin time	11.3 sec/10.9 control	(10.6–12.6)
Renal function		
Creatinine clearance	93.7 ml/min	(77–116)

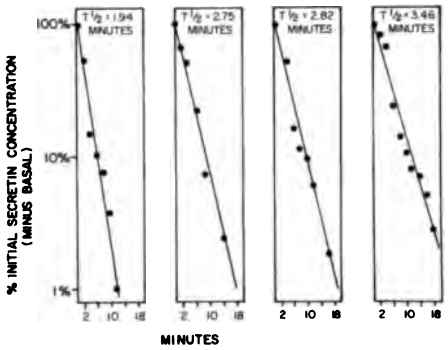

FIG. 1. Linear regression curves plotted from the logarithm of the postinfusion secretin concentrations minus basal expressed as a percentage of the initial concentration at time 0 (x axis) with time in minutes shown on the y axis.

peak bicarbonate output ($r = -0.55869$) or volume ($r = 0.31994$). However, when secretin half-life was compared to weight a negative correlation was found ($r = -0.91354$).

Discussion. The mean secretin half-life values obtained in this study of cirrhotics and healthy volunteers are similar to values reported for canine subjects (8, 9) and to values which we have previously reported in man (6).

We found a negative correlation between secretin disappearance half-life and body weight in the cirrhotic group. This result differs with the findings of Khalil *et al.* (10) which showed a positive relationship in healthy animals. Although the method which they reported for calculating half-lives differs from the usual method used in this report, it does not explain the differing results (personal communication). Other explanations include (i) dissimilar animal species, (ii) dissimilar hepatic and pancreatic function in the two reports, and (iii) small study sample sizes.

The fact that the mean secretin-half lives of the patients with cirrhosis were not significantly different from those of our healthy controls suggests that mechanisms other than altered secretin catabolism are responsible for the hypersecretion seen in response to a secretin test in cirrhotic patients. These results are consistent with recent experimental evidence that the liver may not be a site of significant degradation of either endogenous or exogenous secretin (9). Other potential explanations for pancreatiocobiliary hypersecretion in cirrhosis include:

(i) pancreatic and/or biliary ductal hyperplasia resulting in a larger volume response to a standard secretin stimulus (11),

(ii) an altered pancreatic or biliary secretory threshold to exogenous secretin,

(iii) secretin-induced release of a pancreatic secretory agonist with liver-dependent catabolism, or

(iv) dissociation of biologic and immunoreactivity.

Identification of the mechanisms responsible for the pancreatic and biliary hypersecretion seen in response to exogenous secretin in cirrhotics will require further investigation.

TABLE II. SECRETIN HALF-LIVES IN FOUR PATIENTS AND FIVE HEALTHY CONTROLS AND r VALUES OBTAINED FROM THE LINEAR REGRESSION ANALYSIS

	$t_{1/2}$ (min)	r
Patient		
1	2.75	0.94246
2	3.46	0.98023
3	1.94	0.98140
4	2.82	0.93162
Mean	2.74 ± 0.31 (SEM) min	
Controls		
1	2.77	0.92300
2	2.13	0.96693
3	2.05	0.92911
4	2.37	0.93256
5	4.27	0.99464
Mean	2.72 ± 0.41 (SEM) min	

[a] Using Student's t test for unpaired samples, the mean half-life for the patients does not differ from control ($t = 0.41915$; $0.7 > P > 0.6$).

Summary. The hypothesis of prolonged secretin half-life was examined in four cirrhotic patients who had pancreatic and biliary hypersecretory responses to exogenous secretin. The mean serum half-life of secretin from these patients was 2.74 ± 0.31 (SEM) min and did not differ significantly from a group of five healthy volunteers whose secretin half-life was 2.72 ± 0.41 (SEM) min ($P > 0.1$). In addition, the secretin half-lives correlated inversely with body weight in the cirrhotic patients. These data are consistent with the hypothesis that mechanisms other than altered secretin disappearance half-life are responsible for the hypersecretion seen in response to a secretin test in cirrhotic patients.

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Calcium in Smooth Muscle from the Opossum Esophagus (40681)

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In the distal esophagus of the opossum, the longitudinal and circular layer of the esophageal body and the sphincter are smooth muscle. These muscles contain intrinsic nerves that can be stimulated electrically. Such stimulation by an electrical field leads to contraction in the body of the esophagus, whereas it leads to a relaxation of the high resting tension (or tone) in muscle from the sphincter. Neurogenic contractions in muscle strips from the body of the esophagus are more sensitive to reduction of calcium in the bathing medium than are both the tone and the neurogenic relaxation in strips of sphincter muscle (1). This suggests that the source of calcium which maintains tone in the lower esophageal sphincter is different from the one which allows contraction of muscle from the esophageal body. To investigate this possibility, we compared the content and efflux of calcium in transverse muscle strips cut from the lower esophageal sphincter to that in transverse muscle strips cut from the body of the opossum esophagus.

Methods. The esophagus with a cuff of stomach was removed from mature opossums under barbiturate anesthesia (2). The specimen was opened along the lesser curvature of the stomach and pinned flat on a wax block in a dissection bath. The bath was gassed with 95% O_2–5% CO_2 and its temperature was kept at 36°. The bathing solution contained 15 mmole/liter glucose and (in meq/liter): sodium 139, potassium 4.6, magnesium 2.2, chloride 125, bicarbonate 22, phosphate 3.5, and sulfate 2.3. The calcium concentration in the bathing solution was 2.5 meq/liter. This is about half the concentration generally used (1, 3). This change is based on our finding of this concentration of ionized calcium in the venous blood of five opossums.

Muscle strips, about 2 mm wide and 10 mm long, were cut from four segments of the smooth muscle part of the esophagus. Segment I is the segment of esophagus where the dark red of the proximal striated muscle has just given way to the pale pink of the distal smooth muscle. Segment IV is the segment that constitutes the lower esophageal sphincter. Segments II and III are equally spaced between Segments I and IV. According to a recent study, the thickness of the longitudinal muscle layer remains about the same throughout the entire esophagus. The circular muscle layer doubles in thickness at the lower esophageal sphincter, largely due to the intervention of strands of connective tissue (4). Strips were briefly attached to a force transducer and exposed to an electrical field. All strips from Segment IV showed the tone that characterizes the sphincter, and they relaxed during stimulation. All strips from the body Segments I, II, and III showed "off responses," a phasic contraction after termination of the stimulus that is characteristic of the circular smooth muscle of the opossum esophagus (1). The period beginning with removal of the esophagus and ending with this testing of function never exceeded 30 min.

For determination of tissue calcium content, strips were blotted, weighed, dried in a vacuum, and reweighed. This was followed by ashing at 475° and dissolving the ash in acid. Complete dissolution was obtained by adding 1 ml of concentrated HNO_3 and 0.5 ml of concentrated HCl and heating in a crucible.

Extraction of vacuum-dried muscle strips with 0.5 *N* nitric acid has been used by us previously for measuring tissue sodium and potassium (2). It has been used by others for determination of calcium in smooth muscle (5). However, in four preliminary experiments, we found that calcium content determined by HNO_3 extraction was on the average 20% lower than when ashed muscle was completely dissolved in HNO_3 and HCl. In five additional experiments, we also showed that calcium content was not different when the strip was processed immediately and when it was processed after a period of in-

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cubation in the bath. Calcium content was 2.7 ± 0.2 mmole/kg in strips processed immediately and 2.5 ± 0.2 mmole/kg 2 hr after incubation. All subsequent determinations were done after 2 hr of incubation. Calcium content was measured by atomic absorption spectrophotometry (Perkin–Elmer, Model 303, Norwalk, Conn.) after diluting the samples with distilled water and adding acidic lanthanum (final concentration = 0.1% lanthanum and 0.5% HCl). All glassware and crucibles were acid-rinsed. Duplicate assays were performed on all specimens. Tissue calcium content is expressed in millimoles per kilogram of wet muscle; dry muscle weight is on the average 20% of wet muscle weight.

In nine animals strips were incubated for at least 2 additional hr after labeling of the bath solution with ^{45}Ca. Strips were then removed, blotted, and weighed before beginning the efflux study. For determination of Ca^{2+} efflux, strips were pinned into the opening of a small-bore plastic tube, through which a steady stream of 95% O_2–5% CO_2 was maintained. Strips were dipped for exactly 2 min into each of 15 vials containing 2 ml of unlabeled bathing solution gassed with 95% O_2–5% CO_2 and kept at 36°C.

At the end of the washout period, the strips were again blotted, weighed, and then dissolved by shaking them overnight in glass vials containing 3 ml of a tissue solubilizer (NCS, Amersham/Searle, Arlington Heights, Ill.). Radioactivity in the dissolved samples was determined by liquid scintillation counting after adding 15 ml of scintillant (ACS, Amersham/Searle, Arlington Heights, Ill.) and 0.5 ml of a mixture of EDTA in trichloracetic acid. The amount of ^{45}Ca removed during each 2-min period and the amount remaining at the end of the washout procedure are expressed as a percentage of the total calcium uptake by the tissue. All values are expressed as the mean (X) ±1 SEM. Differences between the four esophageal segments were considered significant when the P value from the paired t test was less than 0.05.

Results. Total tissue calcium content. The calcium content is uniform in the four esophageal segments. The total tissue calcium content in the four esophageal segments (in mmole/kg of wet muscle) is: 2.7 ± 0.1 in Segment I, 2.8 ± 0.3 in Segment II, 2.6 ± 0.3 in Segment III, and 2.6 ± 0.3 in Segment IV after 2 hr of incubation in the Krebs medium.

Washout of calcium-45. Efflux from all esophageal segments is rapid during the first three periods with a halftime of 5 min. Efflux is slow during the subsequent 12 periods with a halftime of 29 min. In all nine animals tested, efflux from sphincter muscle is slower than efflux from the body of the esophagus, and consequently a greater fraction of the total ^{45}Ca uptake remained in sphincter muscle than in muscle from the body of the esophagus. Figure 1 shows an example of an efflux curve from sphincter muscle and from a strip of more proximal muscle in one animal. The mean percentage efflux during all 15 periods is given in Table I.

There is no significant difference in weight of strips from the four esophageal segments (see Table I). Also, there is no statistical correlation between strip weight and fractional efflux during any period ($r < 0.5$; $P > 0.05$).

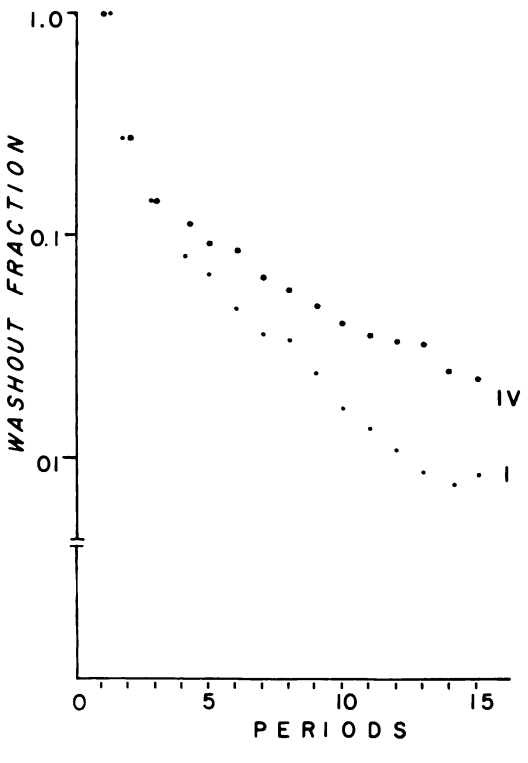

FIG. 1

TABLE I. MEAN PERCENTAGE EFFLUX FROM FOUR SEGMENTS OF ESOPHAGEAL SMOOTH MUSCLE (N = 9)

	Segment			
Period	I (Proximal) (133 ± 17 mg)	II (Middle) (127 ± 20 mg)	III (Distal) (141 ± 16 mg)	IV (LES) (156 ± 18 mg)
1	47.4 ± 3.4	47.0 ± 2.7	45.4 ± 3.5	40 ± 1.5*
2	12.4 ± 1.1	12.4 ± 0.6	13.1 ± 0.7	9.9 ± 0.3*
3	6.7 ± 0.7	6.8 ± 0.6	7.8 ± 0.7	6.0 ± 0.1
4	4.7 ± 0.4	4.8 ± 0.4	5.3 ± 0.2	4.4 ± 0.1
5	3.6 ± 0.2	3.8 ± 0.4	3.8 ± 0.4	3.5 ± 0.1
6	2.9 ± 0.3	2.7 ± 0.2	3.1 ± 0.3	3.2 ± 0.2
7	2.3 ± 0.2	2.3 ± 0.1	2.2 ± 0.2	2.4 ± 0.1
8	2.0 ± 0.1	2.2 ± 0.2	2.1 ± 0.3	2.1 ± 0.1
9	1.7 ± 0.1	1.9 ± 0.3	1.5 ± 0.3	1.9 ± 0.1
10	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.3	1.5 ± 0.1
11	1.3 ± 0.1	1.4 ± 0.2	1.3 ± 0.2	1.5 ± 0.1
12	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	1.4 ± 0.01
13	1.2 ± 0.3	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1
14	1.0 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	1.2 ± 0.1
15	0.8 ± 0.1	0.3 ± 0.1	0.8 ± 0.1	1.0 ± 0.1
Tissue	9.7 ± 1.3	9.7 ± 0.9	9.5 ± 2.0	18.5 ± 1.4*

* Refers to values where sphincter strips differ significantly from body strips. There was no significant difference between any two segments of the body of the esophagus.

Discussion. Our findings indicate that the total amount of cellular calcium is similar in the tonic muscle from sphincter and in phasic muscle from the body of the opossum esophagus; but muscle of the latter type exchanges calcium more readily with the bathing solution.

In the opossum, the tissue potassium content of sphincter muscle is substantially lower than that of muscle from the esophageal body. This is due to a lower cellular potassium concentration in sphincter muscle. In the cat, a gradient of tissue calcium content has been described in small bowel muscle (5). In contrast, our findings indicate that tissue calcium content in the smooth muscle segment of the opossum esophagus does not depend on location. Because of the high contribution of extracellular calcium to the total tissue calcium content, it is, however, possible that small differences in cellular calcium content are missed by our method (2, 3). Different methods such as the ones using La^{3+} or $^{40}Ca^{2+}$–$^{45}Ca^{2+}$ (6) could therefore still demonstrate differences of cellular calcium content between esophageal body and sphincter muscle.

Sphincter muscle exchanges calcium with the bathing solution less rapidly than muscle from the body of the esophagus so that the amount of ^{45}Ca remaining in sphincter muscle is substantially greater than that in body muscle after a prolonged washout. This finding is consistent with our previous observation that muscle from the sphincter shows less functional impairment on reduction of calcium in the bathing solution than muscle from the body of the esophagus. Thus, contractions in the body of the esophagus were effectively abolished 15 min after calcium removal, whereas the tone in the sphincter was only reduced (1).

There could be less efficient exchange of the calcium in sphincter muscle than in muscle from the body of the esophagus because the greater thickness of the sphincter (4) retards diffusion. Thus, extracellular calcium deep in the tissue could account both for the ability of sphincter muscle to maintain its mechanical performance after removal of calcium from the bathing medium and for the greater amount of calcium remaining in the sphincter after washout. There are, however, several arguments against explaining our findings on this basis. The mechanical performance of the sphincter differs from that of the body also in respect to sensitivity to magnesium, strontium, and nitroprusside, all indicating a different action of calcium in sphincter from that in body (1).

A difference in accessibility of the extracellular space also seems unlikely, since all strips are cut so that they were never larger than 2 mm, which is about the maximal wall

ıess. Our experience with markers of ellular space indicates furthermore that nge of extracellular fluid is more than omplete in muscle strips of this size ı about 20 min (2). Furthermore, the ter strips were actually lighter than strips in several animals, but initial ut of calcium in these animals was still nd the fraction remaining in the tissue ill larger than in strips from the esoph- body. Lastly, there is no gradient of ısing washout from segments I to III, hough there tends to be an increase of veight due to greater muscle thickness. dingly, there is for none of the washout ls a correlation between strip weight ashout rate.

s surprising that the higher tissue cal- remaining in the sphincter at the end of 5 washout periods is largely due to r removal during the first washout pe- the periods which reflect washout from tracellular rather than the intracellular

One explanation is that calcium in ter muscle is partially bound to extra- ır structures. Such extracellular calcium ıg has been shown to change the ap- ıce of cellular calcium efflux in taenia or example (7). Another explanation is arly cellular washout rather than extra- ır washout accounts for the observed nces. It has been found by some that m washout from gastrointestinal h muscle can be separated into two and one late phase. The second phase ı halftime between 4 and 7 min ac- d for much of the washout between 2) min. It was assumed to be due to early ır washout, whereas late cellular wash- d a halftime of between 22 and 29 min ccounted for only a small fraction of vashout (8).

ar two main differences between phasic onic types of gastrointestinal smooth have been described with regard to n storage and activation systems. First, smooth muscle contains a relatively smooth endoplasmic reticulum, which ved to be an important store for intra- r calcium (9). Secondly, verapamil ntially blocks phasic contractions of from the gastric antrum, whereas ni- sside blocks preferentially the tonic contraction of the gastric fundus indicating differences in calcium activation systems (10).

The washout of K^+, Na^+, and Cl^- is different in tonic as compared to phasic vascular smooth muscle (11). Our present findings provide preliminary evidence that there are differences in calcium handling between tonic and phasic types of esophageal smooth muscle as well. The above-mentioned structural differences in the tunica muscularis of the esophageal body and the lower esophageal sphincter (4), however, raise the possibility that these variations in calcium handling result from differences in the arrangement of smooth muscle cells rather than from differences in their individual properties.

Summary. Transverse muscle strips were cut from four segments of the opossum esophagus. Segment I was the segment of smooth muscle bordering on the striated muscle proximally and Segment IV was the distal segment which includes the lower esophageal sphincter. Segments II and III were the two central fourths of the smooth muscle esophagus. The tissue calcium content was determined in strips coming from the four segments after ashing and extraction of strips in concentrated HCl–HNO_3. The calcium content (in mmole/kg wet muscle ± 1 SE) was 2.70 ± 0.1 in Segment I, 2.8 ± 0.3 in Segment II, 2.6 ± 0.3 in Segment III, and 2.6 ± 0.3 in Segment IV after 2 hr of incubation.

After incubation of strips coming from the four segments, efflux of ^{45}Ca was measured during 15 two-min periods. Efflux was rapid at first with a halftime of 5 min and slowed after three periods to a halftime of 29 min. In all nine animals tested, efflux from the lower esophageal sphincter was slower than that from muscle of the esophageal body. Thus, calcium content in the smooth muscle esophagus does not depend on location. However, tonic esophageal smooth muscle (sphincter) exchanges calcium less rapidly with the bathing medium than the phasic-type smooth muscle from the body of the opossum esophagus.

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idosis, Hypercalcemia, and Hyperphosphatemia in Rat Fetuses near Term and Effects of Maternal Acid/Base Loading (40682)[1]

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ıost mammalian species examined, the of calcium (1–4) and of phosphorus (5– the blood of the fetus at term exceed of the dam. The difference in serum n between mother and fetus may, in e explained by the observation that the ıting level of this element in the dam ı the final stages of gestation (5, 8, 9). lition, in the many mammals investi- the level of calcium in the blood of the ıas been shown to increase as termina- f pregnancy is approached (10–12). Fi- in the rat, fetal phosphatemia has been to parallel the rise in blood calcium in ıt days before birth (13).

have previously demonstrated that rat ; are acidotic near term, and that *in* hypercalcemia and early neonatal hy- emia are associated with low blood pH ɔrmalization of blood pH, respectively n view of this apparent association at nd immediately after birth, the present ; were undertaken: (i) to investigate er in the final stages of gestation fetal pH shifts concurrently with reported ions in the levels of calcium and phos- ; and (ii) to test the hypothesis that es in the circulating levels of calcium ıosphorus are causally related to blood the developing mammal *in utero*, by aggravating or alleviating the acidotic f the conceptus by the maternal admin- ɔn of ammonium chloride or sodium onate, respectively.

erials and methods. Four- to eight- -old pregnant albino rats and their off- were used. The adult animals were ıined on Purina laboratory chow and ter *ad libitum* and housed in a temper- ɔntrolled room illuminated on a 14- t/10-hr-dark cycle.

rder to obtain females at a known time of gestation, the animals were caged with males for a single overnight period. The day of mating was counted as Day 0, and experiments were terminated at preselected times during the last week of pregnancy.

Studies on fetuses in the final week of gestation. Beginning on Day 17 and then daily through the 22nd day of pregnancy, groups of animals were handled as follows:

The gravid female was lightly anesthetized with ether, a laparotomy was performed, and the pregnant uterus was exposed. Fetuses were sequentially bled and excised until the entire litter was sampled. Fetal blood was taken from the axilla while the placental and umbilical circulations were intact. Tail blood was drawn from the mother at the beginning and end of each experiment.

Studies on offspring of pregnant animals receiving either ammonium chloride or sodium bicarbonate orally. Pregnant animals were divided into three groups. Beginning on the 17th day of gestation, tap water was removed and the following substitutions were made. One group of animals was given a solution of 1.5% NH_4Cl, the second group received a 1.5% solution of $NaHCO_3$, and the remaining rats were given distilled water. Animals were allowed to drink freely and fluid intakes were monitored.

All experiments were terminated on the 22nd day of pregnancy and blood samples were obtained as described above.

Determination of blood pH, respiratory gases, total calcium, and inorganic phosphorus. Samples for determination of pH and respiratory gases were collected in heparinized Natelson blood pipets and kept in an ice bath until analyses were performed. Elapsed time between the taking of samples and their determination was between 1 and 4 hr. Measurements were made with a Corning model 160 pH/blood gas system.

Serum obtained from additional blood samples was analyzed for total calcium by

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atomic absorption spectrophotometry according to the method of Trudeau and Freier (15) and for inorganic phosphorus using the micromethod of Chen *et al.* (16).

Individual blood samples from older fetuses were used whenever adequate. Pooling of samples was necessary in the case of younger fetuses; however, even with pooling, amounts sufficient for pH and respiratory gases were not always available on the earliest days investigated.

Statistical analysis. Statistical evaluation was performed by the Student's *t* test for means.

Results. Blood pH, respiratory gases, calcium, and phosphorus in the last six days of gestation. Calcium and phosphorus. Maternal and fetal blood calcium levels are similar when first examined on Day 17 but beginning on Day 18 they shift in opposite directions with time (Fig. 1a). In the fetus, blood calcium declines sharply from Day 17 to 18 and subsequently increases until term. In the dam, calcium remains fairly constant from Day 17 to 19 (9.5–9.6 mg/100 ml) and then gradually declines to mildly hypocalcemic levels (8.75 mg/100 ml).

The difference between fetal and maternal blood phosphorus ranges between 3 and 5 mg/100 ml at each time point examined (Fig. 1b). Between Days 17 and 19 serum phosphorus initially declines and then rises in both the fetal and maternal organisms. Beyond Day 19, fetal phosphorus steadily increases, whereas maternal phosphorus falls significantly ($P < 0.001$) and remains depressed in the final days of pregnancy.

pH. The small size of blood samples obtainable from 17-day-old fetuses precluded pH determinations at this stage.

Relatively small but statistically significant decrements in maternal blood pH (Fig. 1c) were noted between Days 18 and 19 ($P < 0.001$) and between Days 20 and 21 ($P < 0.05$). By contrast, the fetus showed marked declines ($P < 0.001$) in pH between Days 19 and 20 (7.31 to 7.20) and between Days 21 and 22 (7.16 to 6.96) resulting in a state of pronounced acidosis.

Blood respiratory gases. Because analysis of blood gases requires an even larger sample than that needed for pH determinations, data on fetal pCO_2 and pO_2 could not be obtained for Days 17 and 18. The data presented in Fig. 1d, therefore, represent findings in animals from Day 19 through Day 22 plus Day 18 values of mother rats for comparison with maternal pH values at that time. Also, it should be pointed out that since blood from the axilla (fetus) and tail (dam) represents

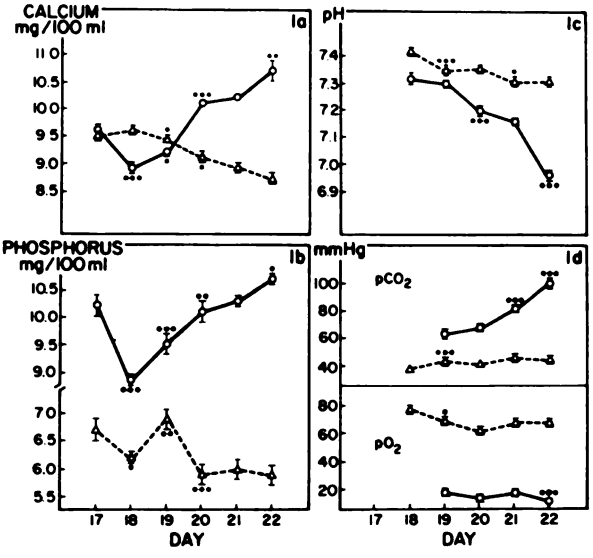
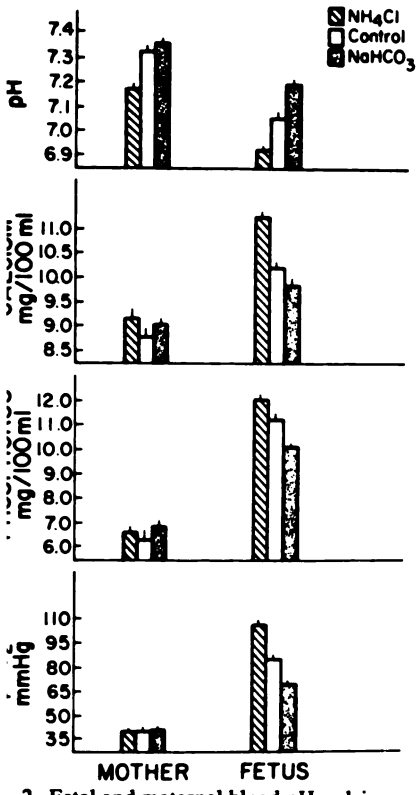


FIG. 1. Fetal and maternal blood pH, calcium, phosphorus, and respiratory gases in the last week of gestation. Each point represents the mean ± SE of either 21–52 maternal or 20–100 fetal samples. (O——O) Fetuses; (Δ- - -Δ) dams. For five of the mean values shown, the standard error is too small to be depicted. Asterisks indicate data which are significantly different from the preceding day: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

l arterial and venous samples, pCO_2 is r and pO_2 lower than would be expected eterminations been done solely on ar- blood.

he maternal organism, small but statis- significant ($P < 0.001$) increases in and a decline in pO_2 occur between 18 and 19. The elevated pCO_2 correlates with the fall in blood pH previously at this time. From Day 19 through no significant alterations in maternal respiratory gases are seen.

ontrast, in the fetus, beginning on Day d continuing through to term, pCO_2 ises in a linear fashion with highly sig- nt ($P < 0.001$) increments occurring on st 2 days of gestation. During the period ined fetal pO_2 remained low with a sig- nt ($P < 0.001$) decrease noted between 21 and 22.



. 2. Fetal and maternal blood pH, calcium, phos- , and pCO_2 on Day 22 of pregnancy. Gravid s were offered 1.5% NH_4Cl, 1.5% $NaHCO_3$, or d water in place of tap water on the preceding 6 ach bar represents the mean ±SE of 15–45 sam-

Administration of ammonium chloride or sodium bicarbonate to pregnant rats during the last six days of gestation (Fig. 2). Administration of NH_4Cl resulted, at term, in a highly significant ($P < 0.001$) depression of maternal blood pH whereas blood pH of $NaHCO_3$-treated dams was unaffected. Neither group of treated females, however, demonstrated any significant alterations in blood calcium, phosphorus, or respiratory gases.

In marked contrast to findings in rat mothers, the fetuses of alkali- or acid-treated dams were profoundly affected. When compared to control values, the blood of unborn offspring of NH_4Cl-treated mothers showed a pronounced decrease in pH (to 6.94) in concert with marked elevations of calcium, phosphorus, and pCO_2. Fetuses of bicarbonate-fed dams had blood with a substantially higher pH (to 7.20), lowered pCO_2, and decreased levels of calcium and phosphorus. All changes in fetal blood were highly significant ($P < 0.001$).

pO_2, not shown in Fig. 2, was unaltered in both mothers and their unborn offspring.

Discussion. At term the mammalian fetus has been reported to be hypercalcemic (1–4) and hyperphosphatemic (5–7), conditions which developed over a period of time in the final stages of gestation (10–13). The present report confirms the gradual buildup of calcium and phosphorus in fetal blood and demonstrates, in addition, that as circulating levels of these elements rise, blood pH falls.

Newly born animals have been reported to be acidotic, a condition which has been attributed to impaired placental circulation during the course of labor and delivery (17, 18), or, alternatively, to a buildup of lactic acid as a result of anaerobic metabolism (19). The acid/base status of the newborn may well be affected as a result of the delivery process; however, from the present study, it appears that like hypercalcemia and hyperphosphatemia, acidosis progressively develops *in utero*. Since blood levels of lactic acid are high at birth (19, 20), the accumulation of acid products of metabolism *in utero* probably contributes to the acidosis noted following delivery, and to the developing acidosis in the fetus in the last stages of gestation. However, a number of investigators have reported that fetal pCO_2 is elevated (14, 21–24)

and, in the present studies, was seen to increase steadily as term was approached. *In utero* acidosis then may well be both metabolic and respiratory in origin.

The present findings on fetal blood pH and calcium are in line with our earlier observations (14) in rat fetuses at term, and in neonates, where it was seen that high levels of blood calcium were associated with an elevated pCO_2 and acidosis *in utero*, and that with the onset of respiration following birth, serum calcium declined following a time course which paralleled increases in and ultimately normalization of blood pH. An association between pCO_2 and/or pH and circulating levels of blood calcium has also been inferred from reports in which blood calcium was increased in adult rats with metabolic and respiratory acidosis (24), in human infants born in a cyanotic state (25), and in fetal rats in response to maternal hypoxia (2). Similarly, blood phosphorus has been shown to be elevated in the presence of lactic acidosis (26), in response to exposure to CO_2 (27), and in the present studies, in association with developing *in utero* acidosis. If concentrations of calcium and phosphorus in fetal blood are related to acid/base status, then *in utero* hypercalcemia and hyperphosphatemia should be aggravated by a further fall in blood pH whereas increasing pH should be palliative. These indeed were the results when either NH_4Cl or $NaHCO_3$ was administered to pregnant females in the last week of gestation.

After 6 days of treatment, the pregnant females themselves responded to acid administration with a lowered blood pH whereas the hydrogen ion concentration in blood of dams receiving $NaHCO_3$ was unchanged. This is in line with observations in adult male rats subjected to acid or alkali loads for almost 1 year (28) but, in the case of $NaHCO_3$, the present results are at variance with short-term studies in pregnant patients wherein infused bicarbonate (29), or hyperventilation in humans (30) and sheep (31, 32), resulted in elevations of blood pH in the gravid females.

In this latter instance, the blood pH of the unborn offspring also rose, an observation which when taken together with the report of metabolic acidosis in laboring patients and their offspring in response to an infusion of fructose (33) lead to the conclusion that the acid/base status of the mother is readily reflected in the developing organism *in utero*.

Our findings in dams and offspring of the NH_4Cl group confirm this, but another explanation is required to account for the increased pH noted in fetuses of bicarbonate-treated mother rats who themselves appeared to be in acid/base balance. The cases mentioned earlier wherein both maternal and fetal pH were elevated involved bicarbonate infusion or hyperventilation in a short time frame (approximately 2 hr prior to delivery), possibly an inadequate period for compensation on the part of the female, and in these cases the prenatal animals do reflect the imbalance present in their mothers. In our hands, however, the extended period (almost 1 week) over which bicarbonate was administered was apparently of sufficient duration for the pregnant females to adjust to the base load and present with a normal blood pH at term. In spite of this, their offspring had blood pH levels higher than controls. Sodium bicarbonate is presumed to readily pass to the fetus by diffusion (34), and it would appear that, in contrast to the adult, the immature organism is incapable of compensating for the base load, and at birth is significantly less acidotic than its untreated counterparts.

It has been repeatedly demonstrated that the concentration of calcium in fetal blood is remarkably constant despite profound alterations in circulating levels of this element in the maternal organism. Thus fetal serum calcium is unaffected by maternal hypercalcemia resulting from administration of parathyroid hormone (35, 36), vitamin D (36), or calcium salts (37, 38) and is relatively unchanged when the mother is hypocalcemic as a result of an infusion of EDTA (38) or following removal of the maternal parathyroid glands (39). In contrast, in the present studies, the offspring of normocalcemic and normophosphatemic mothers had significantly increased or decreased levels of calcium and phosphorus in apparent response to shifts in the pH of fetal blood.

From the present and previous report (14) it would appear that before birth, circulating levels of calcium and phosphorus are related to the acid/base status of the organism *in*

Acid loading of the pregnant female vates naturally occurring fetal acidosis ccentuates the attendant hypercalcemia hyperphosphatemia; administration of ɔonate is palliative and reduces the se- of the derangement in each parameter. ıally, if, as we suggested earlier, neonatal :alcemia is associated with correction of ıcidosis, then decreasing the severity of *ro* acidosis should modify the degree of :tion necessary, and the extent of the n blood calcium, following birth. No nation is currently available on the mineral status of early neonates of ɔonate-treated mothers. However, in the :s where $NaHCO_3$ was infused into fe- patients in labor (29), the infants were ibed as being in better acid/base and al condition than were their counter- born to untreated mothers.

nmary. In the rat fetus, under normal tions, blood calcium, phosphorus, and rise, and pH declines, during the last 6 of gestation. Little or no correlation is between changes noted in the maternal ism and those occurring in her unborn ing.

ıl administration of NH_4Cl to pregnant es results in a lowering of maternal pH, with no change in blood calcium, ɔhorus, or respiratory gases. $NaHCO_3$ ıistration does not alter any of these ıeters. In contrast, profound changes ɔserved in the fetuses of both acid- and -treated dams. With NH_4Cl feeding, fe- l falls while blood calcium, phosphorus, CO_2 rise; with $NaHCO_3$ administration, blood pH increases and serum calcium, ɔhorus, and pCO_2 decline.

ese observations point strongly to an ıation between fetal blood pH and cir- ng levels of calcium and phosphorus, uggest that the levels of these elements ɔe, in part, determined by the acid/base of the organism *in utero*.

authors wish to express their appreciation to Dr. . Smith and Mrs. Donna Breidenbach for their ıd for making available facilities at Children's ıl where all pH and blood gas determinations ɔne.

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Effect of Fat-Enriched Diet on the Incidence of Spontaneous Mammary Tumors in Obese Mice (40683)[1]

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nmary. C3H/HeJ mice show a high in-
ce of spontaneously occurring mam-
tumors. Mice made experimentally
: were observed to have an earlier ap-
nce and a greater incidence of tumors
were the normal-weight mice. A fat-
hed diet increased the observed tumor
ence in normal-weight mice to about
of obese mice given a regular diet, while
enriched diet further enhanced the al-
augmented observed yield of mam-
tumors in the obese mice given a regular
Statistical analysis supported the de-
ence of tumor incidence and appearance
on the type of diet. The calories con-
d by normal mice on a fat-enriched diet
onsiderably below that of obese mice on
ular diet, yet produced about the same
r incidence. A specific effect of the fat-
hed diet is suggested.

gh caloric diets, particularly those mod-
to include a high portion of fat, have
reported to promote or enhance the
rence of several types of tumors in var-
pecies of animals (1–3). Mice, force-fed
hance weight gains, show an increase in
rs. In contrast, dietary restriction de-
or inhibited the formation of sponta-
sly occurring or experimentally induced
rs (4–6). In the present study virgin fe-
mice were made obese by a single injec-
of goldthioglucose (7–16). These mice
ed weights of 50–60 g compared to con-
of 25–30 g and showed an increased
ncy to tumor formation. This was evi-
d by an earlier appearance and a greater
ence of spontaneously occurring mam-
tumors than found in control mice (8–
The excess weight gain has been shown
: due to increased food intake and to
arily represent adipose tissue (11). These mice can mobilize the excess fat during starvation and regain it on *ad libitum* refeeding (11).

Whether dietary fat has a tumorigenic effect apart from its caloric value has been a matter for speculation for at least three decades (1–17–19). Recently, Chan and Cohen reported that rats on a high fat diet had a significantly greater incidence of mammary tumors than those on a low fat diet, although the diets were isocaloric and weight gains comparable (12). The present study was undertaken to reexamine the possible effect of a fat-enriched diet as distinct from its caloric value on mammary tumor incidence in mice.

Materials and methods. C3H female mice with a spontaneous incidence of mammary tumors were obtained from the Department of Genetics, University of California, at 4 weeks of age and kept virginal throughout the experiment. The animals were housed seven to a cage and allowed free access to Purina Chow during the initial stages of the experiments. At 3 months of age, the mice were randomized into two groups, one of which was left untreated and the other injected with goldthioglucose and allowed to become obese. Mice from both groups were fed either commercial Purina Chow or Purina with added 10% Wesson oil by weight. The obese mice on the fat-enriched diet were pair-fed against the obese groups on regular feed. This was done by averaging the weight in grams of diet consumed by mice on the regular diet twice weekly and feeding fat-enriched diet in the same amounts to the companion group. The same procedure was followed in pair-feeding nonobese animals on the fat-enriched diet against the nonobese on the regular diet. The mice were weighed twice weekly and then inspected by palpation for the presence of tumors and the time of onset noted. All animals were autopsied at the time of natural death or termination of the exper-

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iment. The experiment was terminated when 50% of the mice of each of the four groups had developed tumors. All palpable tumors were verified histologically to be mammary carcinomas.

Results. Table I lists the groups of animals, the number of mice in each group, the number of mice in each group developing tumors within 12 months, and their average age and weight at tumor onset. At the end of 12 months the tumor incidence was 71% for obese mice on a fat-enriched diet; 48% for obese mice on a regular diet; 44% for nonobese mice on a fat-enriched diet; and 29% for nonobese mice on a regular diet. The average times of tumor onset for the four groups were 269, 298, 299 and 338 days, respectively. The average weights at tumor onset were 30.0 and 29.7 for nonobese animals on the regular and fat-enriched diets. For obese animals, the average weight was 53.3 g on the regular diet and 55.6 g on the fat-enriched diet.

Figure 1 presents the observations differently from Table I. It plots the ages of individual mice in the four groups at tumor onset against the cumulative percentage of animals developing tumors. (For example, each animal in a group of 34 was plotted as contributing 3.0% to the cumulative percentage, and the experiment was terminated when 17 mice had developed tumors.) The time at which 50% of the mice in each group had developed tumors was 298 days for obese mice on a fat-enriched diet; 369 days for obese mice on a regular diet; 397 days for normal-weight mice on a fat-enriched diet; and 484 days for normal-weight mice on a regular diet. At 298 days of age, when 50% of the obese mice on a fat-enriched diet had tumors, only 11% of the controls on the regular diet had tumors.

To statistically evaluate the influence of the fat-enriched diet on tumor incidence at 12 months, first the interaction between (i) the treatment effect, i.e., fat enrichment versus no fat enrichment; and (ii) the stratification effect, i.e., presence or absence of obesity, was assessed. According to the exact method for contrasting odds ratios (21), there is essentially no evidence of any interaction (P = 0.47). Consequently, a model may be entertained in which the ratios of the odds for tumor appearance at 12 months, of the fat-enriched to non-fat-enriched populations, are assumed to be identical for both obese and nonobese animals. The Mantel–Haenszel test (20) is then appropriate for assessing the null hypothesis that the common value of the odds ratio is unity, i.e., that there is no treatment effect (22). This null hypothesis was rejected (P = 0.05). Within the obese group alone or in the nonobese group alone, tumor incidence

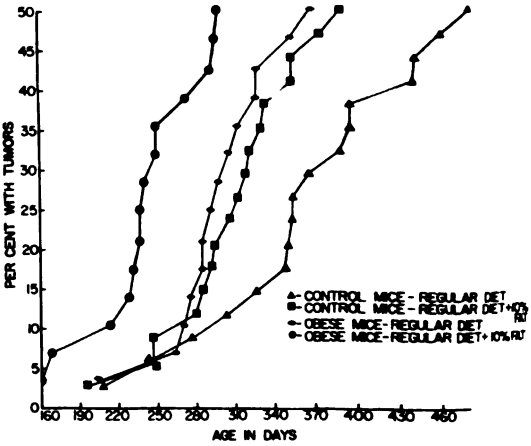
PER CENT WITH TUMORS
AGE IN DAYS
CONTROL MICE - REGULAR DIET
CONTROL MICE - REGULAR DIET + 10% FAT
OBESE MICE - REGULAR DIET
OBESE MICE - REGULAR DIET + 10% FAT

FIG. 1. Plot of ages of individual mice in the four groups at tumor onset against the cumulative percentage of animals developing tumors.

TABLE I. INCIDENCE OF SPONTANEOUS MAMMARY TUMORS IN OBESE AND NORMAL WEIGHT MICE ON REGULAR (PURINA) AND FAT-ENRICHED (PURINA + 10% FAT) DIETS WITHIN 12 MONTHS OF AGE

Group of mice and diet	Number of animals in each group at onset of experiment	Number of mice with tumors	Percentage of mice with tumors	Average weight at tumor onset (g)	Average age at tumor onset (days)
Normal weight, Purina	34	10	29.4	30.0	338
Normal weight, Purina + 10% fat	34	15	44.1	29.7	299
Obese, −Purina	29	14	48.3	53.3	298
Obese, −Purina + 10% fat	28	20	71.4	55.6	269

:en animals given the fat-enriched and
ol diets was not statistically significant.
:ffect of the fat-enriched diet on time of
r appearance (see Fig. 1) was also eval-
l, using Gehan's test. In the nonobese
als the effect was not significant (P =
but in the obese animals the effect was
ïcant (P = 0.01). Combining the two
f animals and adjusting for the influence
esity (in the same way that the Mantel-
szel statistic adjusts for a stratifying var-
, the difference was significant (P =
).

addition, the correlation between the
of tumor appearance and the weight of
als at tumor appearance time in obese
als was assessed in the control and fat-
ıment groups separately, using Spear-
correlation coefficient (24). There is
evidence of correlation in the control
than there is in the fat-enrichment
(P = 0.04 vs P = 0.15). However, these
eparate one-sided inferences may be
ined, using Fisher's method for combin-
values (23) and the summary assess-
is significant (P = 0.05). The correlation
ative, i.e., the later the time of appear-
of a tumor, the lighter the animal at the
There was no significant correlation in
nobese animals.

cussion. Tumor incidence appears to be
nced by at least two experimental fac-
ı the present series. (i) The addition to
et of 10% by weight of fat in the form
esson oil significantly increased tumor
nce in a sample composed of both gold-
ucose-injected, obese and noninjected,
ese mice. (ii) Goldthioglucose injection
resulted in a large observed increase in
nce of tumors compared with nonin-
animals. This occurred whether in-
mice were given a regular or a fat-
ıed diet.

ch of the diet effect seemed to derive
the injected animals and the question
be asked whether an increased inci-
of tumors after goldthioglucose injec-
ould be due to the action of goldthio-
e itself rather than the resultant obesity.
ıced tumorigenesis due to goldthiogluer se appears unlikely since mice made
by goldthioglucose and later reduced
aintained at the weight level of control mice do not show the increased incidence and earlier appearance of tumors noted in the obese groups (10). Also, mice given multiple small doses of gold far in excess of a single toxic dose used to induce obesity do not become obese and do not show an increase in tumor incidence. Hence, it appears likely that hyperphagia resulting from the hypothalamic lesion produced by a single toxic dose of goldthioglucose (13) or the obesity per se would be responsible for an increased tumor incidence in the injected animals.

Purely descriptively, the influence of obesity and of the fat-enriched diet may be related as follows: The fat-enriched diet increased the incidence and shortened the times of appearance of mammary tumors in a combination of normal and obese mice. Goldthioglucose injection in the doses given also resulted in an increased and earlier incidence of tumors. The magnitude of that effect was quite similar to the effect achieved by the fat enrichment of the diet, as may be seen from the two middle curves in Fig. 1 which are almost superimposable. It should be recalled that the food intake of the normal animals on the fat-enriched diet was kept at the level of the controls given normal diet. Hence their caloric intake was only 5% above that of the controls and their average weight at onset of tumors was not different from the 30.0-g average of normal mice given a regular diet. Goldthioglucose-injected animals have a 50% higher food consumption than controls (11) and the average weight at onset of tumors of the injected group on a regular diet was 53.3 gms. Thus both increased food intake (or weight) and the fat-enriched diet are implicated in tumorigenesis.

At this time, the pathogenesis of increased tumor incidence resulting from either obesity, or fat-enriched diet, or both is not known. Recently, Chan and Cohen (12) have reported that overiectomized rats on a high-fat isocaloric diet had palpable mammary tumors whereas there were almost none in animals on a low-fat diet. They attributed this yield of tumors to the high prolactin to estrogen ratio induced in overiectomized rats by a high-fat intake. However, caution is indicated in ascribing the effect of fat-enriched diet to specific estrogenic disturbances known to be involved in the incidence of mammary

tumors, since fat-enriched diet has also increased the yield of other tumors such as hepatomas (14–15) in which a hormonal influence has not been well demonstrated.

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laternal and Neonatal Tissue Lysozyme Levels: Effects of Iron Nutriture in Rats (40684)

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n deficiency anemia is a common nutri- l problem among females of childbear- ge and children (1). In a continuing of experiments in our laboratories, we investigated the effects of maternal iron ency on metabolism in the offspring (2– laternal iron restriction during preg- y and lactation results, in the offspring, merous metabolic alterations including rlipidemia (2, 4), apparently related to ased endogenous triglyceride production nd a decreased ratio of Zn/Cu in some s (3, 5).

n deficiency in the adult female rat has shown to result in increased kidney yme (EC 3.2.1.17) activity when com- l with control animals (6). Lysozyme is teriolytic enzyme discovered by Sir A. ing in 1922 (7) and has been the focus nsiderable interest for more than half a ıry (8). It is considered to be one of al nonspecific immune mechanisms in- d in host defenses (9–11) and is widely buted in vertebrate tissues (12, 13). ified levels of lysozyme have been re- d in a wide range of pathological con- ıs and stimuli (8, 14–17) and nutritional .

animal studies, lysozyme content of var- tissues has been reported to increase ıg vitamin A deficiency (18) and hyper- inosis D (19). Lysozyme was found in ficantly reduced concentration in tears nalnourished preschool-age children, with reductions in secretory IgA and ase (20). In another study of children ıdra *et al.* (21) observed that noninfected ourished children had lower intracellu- ıd plasma lysozyme activity than healthy ols.

nerally, the interrelationships between tional status and lysozyme action merit er study. Our observation of altered kid- ysozyme levels during iron deficiency (6) led us to speculate that the iron-deficient organism, with a lower immune response (22, 23), may require more lysozyme for its antibacterial functions.

In the investigation reported here, we studied the effects of iron restriction on tissue lysozyme levels in maternal rats and their suckling offspring and present previously unreported age-related differences in tissue lysozyme activity.

Materials and methods. Female, nulliparous, Sprague–Dawley CD rats were bred and placed on experimental diets and glass-distilled water, both *ad libitum*, when they weighed approximately 220 g. The iron-deficient diet provided 5 ppm iron and the control 307 ppm iron (Table I). On the second day of lactation, litters were standardized to contain three female and three male pups. The litters were suckled by their dams which remained with them until the 18th day of lactation when both dams and pups were fasted for 4 hr and killed. Thus, the dams were provided experimental diets for a total of 40 days. Sera, kidneys, and spleens were collected from the dams and pups and frozen for future analysis. Milk was collected on Day 18 by palpation when the dams were under Nembutal anesthesia after administration of oxytocin and was also frozen until analyzed. Lysozyme activity was found to be stable for at least 1 month when tissues were stored at −20°. All tissues were assayed within that time. For analysis of lysozyme, 150 mg of spleen and 150–250 mg of kidney were homogenized in 5 ml (spleen) or 10 ml (kidney) of cold 0.066 *M* potassium phosphate buffer, pH 6.24, and centrifuged at 100,000*g* for 1 hr at 2°. In the case of pup tissues, two organs from each litter were pooled for homogenization and subsequent analysis. After centrifugation, the supernatant was used for measurement of lysozyme activity by monitoring the lysis of cell walls of *Micrococcus lysodeikticus* (24).

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Lysozyme rate was expressed as

$$\text{units/mg protein} = \frac{\Delta A\ 450/\text{min}}{0.001 \times \text{mg protein/reaction mixture}}.$$

Serum was used directly for enzyme analysis. The volumes of supernatant and sera used for the assays were within the linear range of enzyme activity. Protein was measured by the method of Lowry *et al.* (25) with bovine serum albumin as standard. Milk was prepared for assay by precipitating 200 μl with 200 μl of 10% TCA and centrifuging; the supernatant was then dialyzed with water overnight at 4°. The samples were lyophilized, reconstituted with 25 μl H_2O, and applied to a lysoplate with *Micrococcus lysodeikticus* agar. The lytic activity of these extracts was measured quantitatively by the radial diffusion-in-gel technique and is expressed as egg lysozyme equivalents as described by Kuettner *et al.* (26). Hemoglobin was determined on tail blood samples by the method of Crosby *et al.* (27), and microhematocrits as described by Wintrobe (28). Statistical significance between group means was detected using Student's *t* test and that between means of dams and their pups by the paired *t* test (29).

TABLE I. COMPOSITION OF DIETS

	Concentration (%)	
	307 ppm Iron	5 ppm Iron
Casein[a]	22.00	22.00
Sucrose	29.70	29.76
Cornstarch	29.70	29.76
Iron-free salt mix[b]	5.48	5.48
Vitamin mix[c]	1.00	1.00
Corn oil[d]	10.00	10.00
Cellulose[e]	2.00	2.00

[a] Vitamin-free casein, Teklad, Chagrin Falls, Ohio.

[b] The level of minerals used is at least 12.5% more than U.S. National Research Council recommendations for lactation (30). Composition of salt mixture (mg/100 g diet): $CaCO_3$, 1680.0; $CoCl_2 \cdot H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 9.8; $MgSO_4 \cdot 7H_2O$, 278.5; $MnSO_4 \cdot H_2O$, 19.0; KI, 0.02; NaCl, 1000.0; K_2HPO_4, 2483.6; $ZnCl_2$, 3.0. $FeSO_4 \cdot 7H_2O$ was added to the supplemented diet in place of sucrose and cornstarch.

[c] Teklad, Chagrin Falls, Ohio. Supplies in mg per kg of diet when added at 1% of diet: *p*-aminobenzoic acid, 110.1; ascorbic acid, 991.2; biotin, 0.4; vitamin B_{12}, 0.03; calcium panthothenate, 66.1; choline, 1433.7; folic acid, 2.0; inositol, 110.1; menadione, 49.6; niacin, 99.1; pyridoxine-HCl, 22.0; riboflavin, 22.0; thiamine-HCl, 22.0. Supplies, in units per kg of diet, when added at 1% of diet: vitamin A-palmitate, 19,824; vitamin D_2, 2203; vitamin E acetate, 121. Cornstarch dilutent QS.

[d] Mazola corn oil, Best Foods, Englewood Cliffs, N.J.

[e] Alphacel nonnutritive cellulose, Chagrin Falls, Ohio, with iron extracted by the method of Houk *et al.* (31).

Results. As indicated in Table II, the mean body weights, hemoglobin levels, and hematocrits of iron-deficient pups were significantly lower ($P < 0.001$) than those of control pups. Similarly iron-deficient dams had lower ($P < 0.001$) hemoglobin and hematocrit levels than did control animals.

The growth retardation in iron-deficient pups and maintenance of normal body weights in dams observed in the present study confirm results of previous studies (2–4). The extent of anemia as determined by hemoglobin and hematocrit values reported here for maternal and suckling rat pups is also comparable to our previously reported values (2,

TABLE II. BODY WEIGHTS, WEEKLY FOOD INTAKES, HEMOGLOBIN LEVELS, AND HEMATOCRITS DURING LACTATION

	Food intake (g) during lactation		Body weights (g) during lactation		Day 18 of lactation		
	Week 1	Week 2	Day 8	Day 15	Body wt (g)	Hemoglobin (g/dl)	Hematocrit (%)
Dams							
5 ppm iron	179 ± 21[a]	192 ± 12	312 ± 6	317 ± 5	311 ± 5	12.8 ± 0.5	40.8 ± 0.6
307 ppm iron	154 ± 11	210 ± 26	325 ± 7	328 ± 9	328 ± 9	18.4 ± 0.3	56.0 ± 1.0
	NS[b]	NS	NS	NS	NS	$P < 0.001$	$P < 0.001$
Pups							
5 ppm iron			12 ± 1	23 ± 1	24 ± 2	4.2 ± 0.2	14.2 ± 0.8
307 ppm iron			15 ± 1	32 ± 3	40 ± 5	11.2 ± 1.2	45.8 ± 2.1
			$P < 0.02$	$P < 0.02$	$P < 0.001$	$P < 0.001$	$P < 0.001$

[a] Mean ± SE for eight rats per group.

[b] Statistical significance determined by Student's *t* test.

he lower body weights and hematologi-
alues of iron-deficient pups are directly
:d to the iron deficiency per se since
rnal food intake and body weights did
liffer between groups (Table II). The
r iron concentration of milk which we
reported for iron-deficient dams (4) also
ibuted to the differences in growth, he-
obin, and hematocrit levels between
ps.

ssue lysozyme levels (Table III) were
l to differ between both dietary groups
etween the maternal rats and their off-
g. There was an overall trend toward
r tissue lysozyme levels during anemia
ubstantial differences in lysozyme activ-
f the different tissues. Renal lysozyme
were significantly higher in the dams
in the pups for both dietary treatments
0.005). However, there were no signifi-
differences in kidney levels of lysozyme
en iron-deficient and control dams or

Lysozyme levels in the spleen differed
respect to both age and iron nutriture.
e spleen the pups had lysozyme levels
ximately twice those found in the lac-
adult females ($P < 0.02$). The pups in
on-deficient group had spleen lysozyme
ty which was almost twice as high as
of the pups in the control group ($P <$
. In contrast to this, dams fed both 5 and
pm iron had similar spleen lysozyme
.

e contribution of spleen lysozyme to the
defense of the suckling pup is thus much
er than in the adult. Effects of iron
iency on spleen lysozyme were seen only
pups where it was elevated about two-
n the deficient pups. Thus, we observed
ficant effects of both age and iron on
n lysozyme content.

the control group, serum lysozyme in
and dams was not significantly different;
ver, in the pups, it was affected by iron
ture. A twofold elevation in serum lyso-
was seen in the iron-deficient pups
ared to the control pups ($P < 0.001$).

vels of milk lysozyme (Table III) were
mined to test the hypothesis that the
deficient dams produced milk with
r lysozyme activity than controls and
this lysozyme was transmitted intact to
era and/or spleens of the pups. This apparently was not the case since there were no significant differences in milk lysozyme levels as measured on Day 18 of lactation. It is possible that earlier during lactation, possibly during colostrum secretion, (8) when milk lysozyme is highest, there were iron-related differences.

Discussion. Lysozyme no doubt plays an important part in the resistance to infection during perinatal life (33). The age-related differences in tissue lysozyme activity reported in this study point to heretofore unrecognized shifts in the lysozyme levels of different tissues during the life cycle. In the adult, the kidney contributes slightly more than it does in the suckling pup. However, splenic lysozyme is considerably higher in the young than in the mature rat. Serum lysozyme apparently remains constant with age and is sensitive to iron nutriture only in younger animals.

In previous studies in our laboratory, we have also observed histopathologies of the spleen and the thymus in iron-deficient pups[1] which suggest depressed lymphopoiesis in both B- and T-cell areas. It is possible that lysozyme, as a nonspecific factor in immunity, is increased in the iron-deficient organism in an attempt to compensate for the depressed potential for cell-mediated immunological response. The elevation in splenic lysozyme activity in pups may be related to a specific, albeit presently unknown, function of the spleen in lysozyme action during the suckling stage. Thus, the pup's spleen may be more sensitive to the iron deficiency than is the maternal organ.

At this point, knowledge of the physiological function of lysozyme is incomplete, and one can only speculate about the significance of lysozyme changes with iron nutriture (6). Jollés has postulated that in addition to lysozyme's bacteriostatic activity, the peptidoglycans formed by the digestive action of lysozyme on bacterial cell walls may exert an adjuvant or immunostimulating action (11). In the iron-deficient rats with less immunocompetency, this immunostimulating activity of lysozyme may assume greater importance.

[1] Rothenbacher, H., Sherman, A. R. Target organ pathology in iron-deficient suckling rats. (Submitted for publication.)

TABLE III. TISSUE LYSOZYME ACTIVITIES

	Kidney				Spleen				Serum				Milk	
	Dam		Pup		Dam		Pup		Dam		Pup			
	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron
Organ wet weight (g)	1.111[a] ±0.036	1.100 ±0.052	0.187 ±0.014	0.219 ±0.020	0.507 ±0.027	0.537 ±0.036	0.061 ±0.008	0.154*** ±0.021						
Protein (mg/g tissue) or (mg/ml serum)	43.15 ±2.45	45.70 ±2.55	59.74 ±6.85	39.98* ±4.26	59.95 ±3.14	57.47 ±1.98	49.30 ±2.24	45.96 ±1.68	6.81 ±0.12	6.45 ±0.20	7.07 ±0.34	6.59 ±0.19		
Lysozyme (unit/mg protein)	64.52††† ±4.45	59.82††† ±6.03	47.56 ±1.92	44.21 ±4.66	5.43††† ±0.85	5.46† ±0.48	20.56 ±2.51	10.55** ±1.78	3.82 ±0.28	4.25 ±0.25	8.81 ±0.63	4.40*** ±0.53	2.4[b] ±0.6	2.7 ±0.5

[a] Mean ± SE for eight rats per group for kidney, spleen, and milk. Six rats per group for serum.

[b] Milk lysozyme activity expressed as egg white lysozyme equivalents (26).

* Statistical significance between means of iron-deficient and of control pups: $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, determined by Student's t test.

† Statistical significance between dams and their pups in same treatment: $P < 0.02$, †† $P < 0.005$, ††† $P < 0.001$, as determined by paired t test.

It is also possible that in the iron-deficient animal, lactoferrin, an iron-binding protein which like lysozyme is part of the locally secreted mucosal defense system (34), is less effective and lysozyme action increases as a compensation. Possibly the increased level of lysozyme activity in the iron-deficient animal is related to the reported increase in antibacterial action of lysozyme when the chelating agent EDTA is added to *in vitro* cultures (35). Chelating agents bound to trace minerals such as iron may have created an *in vitro* deficient state somewhat comparable to the dietary iron deficiency in our investigations.

Summary. The results of this investigation indicate that iron deficiency in the suckling rats alters immunodevelopment with respect to lysozyme activity. It was also found that suckling and mature rats have different tissue distributions and activities of lysozyme. The health and biomedical implications and significance of these findings are unclear at present.

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calorie	cal	millimeter	mm
centimeter	cm	milliosmole	mOsm
counts per minute	cpm	minute	min
cubic centimeter	cm^3	molal (concentration)	*m*
Curie	Ci	molar (concentration)	M
degree Celsius (Centigrade)	°C	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	diam	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
inch	in.	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
microliter	µl	square centimeter	cm^2
micrometer	µm	square meter	m^2
milligram	mg	subcutaneous	sc
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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 375–379 (1979)

t of Acetazolamide on Renal Tubular Bicarbonate Reabsorption in Newborn Dogs (40685)[1]

LEONARD I. KLEINMAN AND THERESA A. DISNEY

University of Cincinnati, College of Medicine, Department of Pediatrics, Cincinnati, Ohio 45267

previous report (1) we demonstrated nder conditions where isovolemia was ined, there was no renal tubular max- (T_M) for bicarbonate reabsorption in rn dogs, and that as much as 50 μeq of onate per milliliter glomerular filtrate be reabsorbed. Moreover, when car- anhydrase was inhibited by 50 mg/kg olamide, only 4.5 μeq/ml g.f.r. of bi- iate reabsorption was inhibited, an it less than 20% of the total bicarbonate rption, indicating that the major por- renal bicarbonate reabsorption occurs ndent of the effects of carbonic anhy- A recent report by Mathisen *et al.* (2), er, suggests that the dose of 50 mg/kg tazolamide may have been insufficient npletely inhibit carbonic anhydrase. resent study was carried out, therefore, blish a dose–response relationship for olamide in the newborn dog, and to ine whether the conclusions reached earlier paper were valid, or instead, ave been inaccurate due to incomplete tion of carbonic anhydrase.

hods. Studies were carried out on a f 15 mongrel puppies, of either sex, 5– s of age. The animals were anesthetized i.v. injection of pentabarbitol, 25 mg/ d placed on a temperature-controlled g board regulated to maintain rectal rature at 37°. The trachea was exposed nnulated to clear the airway and to positive-pressure ventilation. Poly- ne catheters of appropriate size (PE50 60) were placed in the right external r vein for infusion of ^{3}H-labeled inulin aintenance fluids, in the right femoral or infusion of bicarbonate-enriched in the ascending aorta via the right l artery for recording of blood pressure, descending aorta via the left femoral artery for withdrawal of blood, and in both ureters for collection of urine.

All animals were infused with a maintenance solution containing glucose (50 g/liter), sodium (20 meq/liter), potassium (10 meq/liter), and chloride (30 meq/liter) at a rate of 0.06 ml/min·kg. A priming injection of ^{3}H-labeled inulin, 3–5 μCi/kg, was followed by a constant infusion of labeled inulin at a rate of 0.06 μCi/min·kg. After a 1-hr equilibration period, a 30-min control clearance was taken.

Plasma bicarbonate levels were changed by the exchange transfusion technique described previously (1). This technique permitted large changes in plasma bicarbonate levels without changing body water and sodium since body weight, hematocrit, and plasma sodium concentrations remained constant throughout the experiment. Blood was withdrawn from the femoral artery at a rate of 1 ml/min·kg while bicarbonate-enriched blood was infused simultaneously at exactly the same rate into the femoral vein. Bicarbonate-enriched blood was prepared by separating plasma from the blood of an adult donor dog, dialyzing the plasma against a high-bicarbonate, multielectrolyte solution (Table I), and then recombining the high-bicarbonate plasma and the red cells to a hematocrit identical to that of the experimental puppy.

Every 30 min during the exchange transfusion, renal clearance studies were performed. Calculations of the renal clearances were made from the blood withdrawn during the exchange transfusion and the urine that was collected over that 30-min clearance period.

Following the collection of two to three clearance studies, acetazolamide was administered at various doses to three different groups of animals. During the acetazolamide infusion, the exchange transfusion with high-bicarbonate blood was continued to maintain high plasma bicarbonate levels.

; investigation was supported by the Cincinnati 's Hospital Research Foundation.

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TABLE 1. COMPOSITION OF MULTIELECTROLYTE SOLUTION FOR EXCHANGE TRANSFUSION

	mM
Na^+	140
K^+	5
HCO_3	140
Mg^{2+}	0.85
Ca^{2+}	2.5
Cl^-	10
SO_4^{2-}	0.85

In five puppies acetazolamide was infused intravenously at a priming dose of 5 mg/kg followed by a maintenance dose of 5 mg/kg/hr for the duration of the experiment (usually another 2 hr). Clearance studies were performed every half hour beginning immediately after the administration of the priming dose.

Five puppies received a priming dose of 50 mg/kg acetazolamide followed by a maintenance infusion of 50 mg/kg/hr for 1 to 2 hr. Then these puppies received another acute dose of acetazolamide at 100 mg/kg followed by a sustaining dose of 100 mg/kg/hr for another 2hr. Clearance studies were performed every half hour beginning immediately after the administration of the first dose.

Five puppies received a dose of 500 mg/kg acetazolamide followed by a maintenance infusion of 500 mg/kg/hr for 2 hr and as in the other experiments, half-hour clearance studies were begun immediately after the administration of the priming dose.

Blood and urine pH and pCO_2 were measured with a Radiometer PHM71MK2 acid-base analyzer. Plasma and urine bicarbonate were calculated from the Henderson-Hasselbalch equation using a pK' of 6.1 for blood (3) and of $6.33 - 0.5([Na+] + [K+])^{1/2}$ for urine (4) estimating the ionic strength of urine as the sum of the concentrations of sodium and potassium.

Sodium concentration of plasma and urine samples were determined by atomic absorption spectrometry (Perkin-Elmer). Plasma and urine inulin were determined from samples placed in Instagel solution for scintillation counting (Packard) of tritium. Hematocrit was measured on all blood samples. Blood pressure was measured directly from the ascending aorta with a transducer (Statham) and recorder (Hewlett-Packard).

The g.f.r. was equated with inulin clearance. The amount of bicarbonate filtered was calculated as the product of g.f.r. and plasma bicarbonate concentration. The amount of bicarbonate reabsorbed was calculated as the difference between the amount of bicarbonate filtered and that excreted and expressed as microequivalents per ml g.f.r. Similar calculations were made for sodium. All results were analyzed with standard statistical computer programs available at the University of Cincinnati.

Results. A bicarbonate titration curve of values taken from all of the animals prior to administration of acetazolamide is presented in Fig. 1. As is readily apparent from the figure, no T_M for bicarbonate was found, and as much as 50 μeq bicarbonate could be reabsorbed per ml g.f.r. Except for a few cases, all of the filtered bicarbonate was reabsorbed even when the plasma bicarbonate exceeded 30 meq/liter. On the average, 0.19 μeq/ml g.f.r. of bicarbonate was excreted, amounting to less than 1% of the filtered load (Table II). When acetazolamide was administered, bicarbonate reabsorption fell and excretion increased (Figs. 2 and 3). As can be seen from the bicarbonate titration curve following the various doses of acetazolamide (Fig. 2), there was still no T_M for bicarbonate even with plasma values exceeding 50 meq/liter. At 5 mg/kg of acetazolamide, reabsorption was inhibited less than at the higher doses ($P < 0.01$), but there was no difference among the three higher doses ($P > 0.05$, using analysis of variance). The line of regression

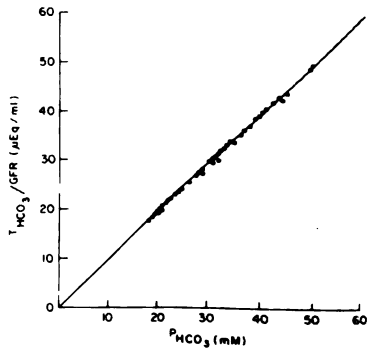

FIG. 1. Bicarbonate reabsorption ($T_{HCO_3/g.f.r.}$) as a function of plasma bicarbonate (P_{HCO_3}) before administration of acetazolamide. The straight line represents complete reabsorption of filtered bicarbonate.

I. BICARBONATE EXCRETION ($E_{HCO_3/g.f.r.}$), ONAL BICARBONATE EXCRETION ($F.E._{HCO_3}$), RACTIONAL SODIUM EXCRETION ($F.E._{Na}$) NBORN DOGS UNDER VARIOUS DOSES OF OLAMIDE

$E_{HCO_3/g.f.r.}$ (μeq/ml)	$F.E._{HCO_3}$ (%)	$F.E._{Na}$ (%)
0.19 ± 0.07[a]	0.72 ± 0.25	0.43 ± 0.09
1.99 ± 0.20	6.12 ± 0.65	2.64 ± 0.27
4.84 ± 0.35	15.80 ± 2.20	4.55 ± 0.30
4.90 ± 0.36	16.30 ± 1.80	5.02 ± 0.35
6.13 ± 0.78	15.25 ± 1.84	5.57 ± 0.84

s are mean ± SE.

the figure is based on the combined m the three higher dose groups and ; 0.96, is not significantly different In other words, the same amount of ate was inhibited by acetazolamide dent of the plasma bicarbonate con- on or filtered bicarbonate load. This l further in Fig. 3 which reveals the orrelation ($P > 0.10$ for all analyses) bonate excretion (representing that of bicarbonate reabsorption inhibited zolamide) and plasma bicarbonate group ($r = 0.17$, 0.24, 0.06, and 0.45 , 50, 100, and 500 mg/kg dose groups, ely) as well as for all the groups d ($r = 0.18$). Also apparent from Fig. there is no difference in bicarbonate n among the three higher dose groups zolamide although bicarbonate excre- wer at the 5 mg/kg dose.

there was no correlation between l bicarbonate and plasma bicarbon- possible to analyze the data from the f the various dosage groups (Table g analysis of variance, no difference rbonate excretion could be found the three higher dosage groups ($P >$ t bicarbonate excretion was lower at g/kg dose ($P < 0.01$). Bicarbonate n at the higher acetazolamide dose veraged 5.3 μeq/ml g.f.r. Fractional ate excretion (excreted divided by and fractional sodium excretion fol- imilar dose–response patterns. At the gher acetazolamide dose levels, the excreted only 15–16% of the filtered ate and 5% of the filtered sodium. It e noted, however, that fractional bi- te excretion was slightly greater at lower plasma bicarbonate levels than at higher levels since filtered load was changing with changing plasma bicarbonate levels while excreted bicarbonate was not. At plasma bicarbonate levels of 25 m*M* fractional bicarbonate excretion averaged 21% while at levels of 40 m*M*, it was 13%.

Discussion. Results from the present study confirm results from our previous studies performed on different animals (1). When a bicarbonate titration curve is performed in the neonatal dog and body fluid volume is maintained constant by exchange transfusion, there is no renal T_M for bicarbonate reabsorption and this reabsorption is essentially complete, at least up to values of 50 μeq/ml g.f.r.

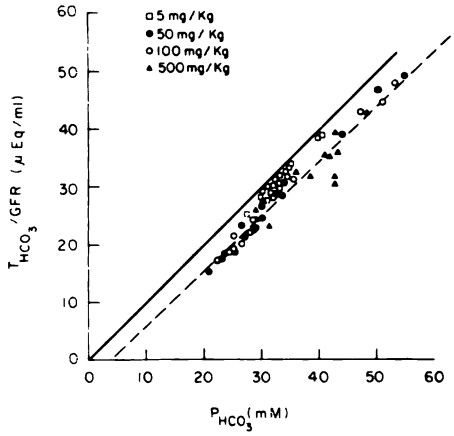

FIG. 2. Bicarbonate reabsorption ($T_{HCO_3/g.f.r.}$) as a function of plasma bicarbonate (P_{HCO_3}) after administration of the various doses of acetazolamide. The straight line represents complete reabsorption of filtered bicarbonate. The dashed line represents the regression equation based on the data from the three highest dosage groups, $y = 0.96x - 3.72$.

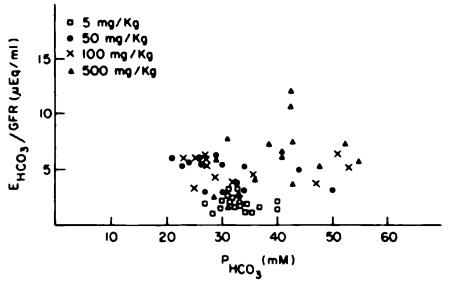

FIG. 3. Bicarbonate excretion ($E_{HCO_3/g.f.r.}$) as a function of plasma bicarbonate (P_{HCO_3}) after administration of the various doses of acetazolamide.

In the earlier study, we wished to investigate the role of carbonic anhydrase in renal bicarbonate reabsorption in the neonatal dog and chose a dose of 50 mg/kg of acetazolamide to inhibit the enzyme. At that time, based on published reports (5, 6), we believed that this dose was sufficient to completely inhibit carbonic anhydrase. However, recent reports have suggested that carbonic anhydrase inhibition may have been incomplete at the acetazolamide dosage used. Mathisen, *et al.* (2) found that in adult dogs under conditions of volume expansion and ethacrynic acid administration, at a dose of acetazolamide of 30 mg/kg, renal bicarbonate reabsorption was 49% of the filtered load but at a dose of 500 mg/kg, it fell to 19% of the filtered load. In the present study at a dose of 50 mg/kg, bicarbonate reabsorption averaged 84–85% of the filtered load and did not change when the dose was raised to 100 or to 500 mg/kg. There are a number of factors that may account for the discrepancies between the two studies. One explanation for the difference may be that mechanisms of renal bicarbonate reabsorption could be different in the neonatal than in the adult dog. Although small differences in bicarbonate reabsorptive mechanisms between adult and newborn dogs cannot be ruled out, it is unlikely that large differences exist for the following reasons. The bicarbonate excretion response of puppies at acetazolamide doses of 5 and 50 mg/kg in the present study could be predicted from the acetazolamide dose–response curve done in adult dogs by Maren (7). Also, the values for the carbonic anhydrase dependent and independent bicarbonate reabsorption in puppies from these and previous experiments, are similar to those obtained by Garg (8) in nonexpanded adult dogs. In those studies benzolamide, when administered at doses calculated to completely inhibit carbonic anhydrase, inhibited the reabsorption of from 4 to 5.7 μeq bicarbonate/ml g.f.r. whereas acetazolamide at the higher dosage levels, inhibited an average of 5.3 μeq bicarbonate/ml g.f.r. in puppies of the present study. Moreover, other studies from our laboratory (9) investigating the interrelationships among bicarbonate, sodium, and chloride reabsorption in the proximal tubule have revealed similarities between newborn and adult dogs.

Another explanation for the differences of results may be related to the different fluid states of the animal between the two studies. For example, animals in the present study were not volume explanded whereas those of Mathisen *et al.* were. In addition, animals in the present study maintained constant fluid balance throughout the experimental procedure. Volume expansion is known to inhibit bicarbonate and sodium reabsorption, (10) which probably accounted for the greater degree of inhibition of bicarbonate reabsorption, even at the lower acetazolamide dosage, in the animals of Mathisen *et al.* (2).

The reason for the large difference in the response at the very high bicarbonate dose between the present study and that of Mathisen *et al.* may be found in the results of earlier studies by Relman *et al.* (11) and Maren (12). Relman *et al.* (11) found that a dose of 500 mg/kg of acetazolamide administered to adult dogs did indeed produce a large increase in bicarbonate and sodium excretion compared to that from an acetazolamide dose of 10 mg/kg. However, when an N^6-methyl analog of acetazolamide was administered at the same high dose of 500 mg/kg, there were also a large increase in bicarbonate excretion. This large bicarbonaturia occurred even though there were only negligible amounts of carbonic anhydrase-inhibiting drug found in the blood or urine since the analog had negligible carbonic anhydrase-inhibiting effect and less than 0.1% of the drug was converted to the active carbonic anhydrase inhibitor, acetazolamide (12). These results with the acetazolamide analog suggest that the increase in bicarbonate excretion from the large dose of acetazolamide was due not to carbonic anhydrase inhibtion but to some other factor. Since the large dose of acetazolamide, as well as its analog, produced a large increase in blood pH (due to the buffer base properties of the drugs), the authors suggested that the change in bicarbonate excretion could have been due to changes in body pH. Unfortunately, in the report of Mathisen *et al.*, there is no data relating to pH changes due to infusion of the large dose of acetazolamide. Since there was no control experiment with a noncarbonic anhydrase-inhibiting analog, there is no certainty that

ir results could not be explained by a
icarbonic anhydrase inhibitory action of
large dose of acetazolamide, perhaps re-
d to body pH changes. In the present
eriment, the exchange transfusion tech-
ue permitted stabilization of pH homeo-
is to the extent that following infusion of
tazolamide at the 500 mg/kg dose, there
a statistically insignificant ($P > 0.05$)
nge of blood pH by only 0.01 units. This
ilization of pH may have prevented the
icarbonic anhydrase inhibitory effect of
large dose of acetazolamide, permitting a
dose–response effect on carbonic anhy-
se inhibition. The present study, therefore,
ports the conclusions of the earlier exper-
nt from this laboratory (1) demonstrating
t at high plasma bicarbonate levels in
expanded puppies, the carbonic anhy-
e dependent portion of bicarbonate reab-
tion accounts for less than 20% of the
renal bicarbonate reabsorption. In ad-
n, in the neonatal dog the dose of ace-
mide of 50 mg/kg is sufficient to com-
y inhibit carbonic anhydrase activity
higher doses caused no increase in bi-
ate or sodium excretion.

mary. Bicarbonate titration curves
termined in 15 neonatal dogs, 5 to 30
age, under conditions of isovolemia
h the administration of various doses
zolamide to inhibit renal carbonic
se. Under each experimental condi-
, no T_M for bicarbonate reabsorption
found. With no acetazolamide, es-
sentially all of the bicarbonate filtered was reabsorbed even at filtered loads of 50 μeq/ml g.f.r. Acetazolamide at a dose of 5 mg/kg inhibited less renal bicarbonate reabsorption than at doses of 50, 100, and 500 mg/kg but there was no difference among the latter three doses. At the higher acetazolamide dose levels, 5.3 μeq of bicarbonate was inhibited per ml g.f.r., amounting to only 12–24% of the total bicarbonate reabsorption (depending upon plasma bicarbonate levels) indicating that the major portion of the renal bicarbonate reabsorption in the neonatal dog is independent of the effects of carbonic anhydrase.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 380–382 (1979)

A Comparison of [^{35}S]Sodium Sulfate and [^{75}Se]Selenomethionine as Platelet Labels for the Assay of Thrombopoietin (40686)[1]

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Mice have been utilized in several previous studies for the assay of a thrombocytopoiesis-stimulating factor (TSF or thrombopoietin). To measure platelet production rates, some workers (1–4) used [^{75}Se]selenomethionine (^{75}SeM) as a platelet label while others (5–11) used [^{35}S]sodium sulfate. Presumably, either isotope would label platelets to the same degree and could, therefore, be used to detect increased thrombocytopoiesis; however to date, the two isotopes have not been compared in the same study. The results show that both isotopes measure the increased thrombocytopoiesis that follows TSF injection, but $Na_2\,^{35}SO_4$ gives significantly greater values than ^{75}SeM after high TSF doses when compared to suitable saline-treated control animals.

Materials and methods. Male C3H mice weighing approximately 25 g were used. Some of the mice were pretreated with a single intraperitoneal (i.p.) injection of rabbit anti-mouse platelet serum (RAMPS) which had been prepared and absorbed as previously described (7).

The TSF used in this study was culture medium from human embryonic kidney cells (Lot WTSF-80, 164 mg of protein/ml). Medium from kidney cell cultures was shown previously to contain high levels of TSF (12). The medium was collected, concentrated, and stored as previously described (11).

TSF was injected subcutaneously (s.c.) two times on Day 5 after RAMPS treatment and again two times on Day 6. On Day 7, either 30 μCi (8) of [^{35}S]sodium sulfate (New England Nuclear, Boston, Mass.) or 1 μCi (3) of [^{75}Se]selenomethionine (Sethotope, E. R. Squibb & Sons, New Brunswick, N. J.) diluted to 0.5 ml in saline was injected intravenously (i.v.) and the 24-hr isotope incorporation into platelets was measured. Using similar schedules, normal mice (mice not injected with RAMPS) were injected with TSF and radioisotopes.

Immediately before killing, mice were injected i.p. with 0.5 ml of heparin–Nembutal solution (heparin, 1000 U.S.P. units; Nembutal, 50 mg; 10 ml of saline). Platelet counts were taken from a single drop of blood obtained from the retroorbital sinus. Approximately 3 min later mice were bled (about 0.5 ml/mouse) by cardiac puncture into plastic syringes containing 1 ml of 1.0% EDTA, and the blood was expressed into plastic tubes. After dilution with additional EDTA solution to a total volume of 3.0 ml, the blood was centrifuged (4.5 min at 450 g) to obtain platelet-rich plasma. Platelet-rich plasma was removed and centrifuged (15 min at 800*g*) to obtain platelet "pellets"; the platelets were washed twice in 1.0% ammonium oxalate and once in saline. The washed platelets were resuspended into 0.35 ml buffered saline and the number of platelets in suspension was determined by use of an Electrozone/Celloscope (Particle Data, Inc.). Other samples of the platelet suspension were used to determine radioactivity. The % ^{35}S and ^{75}Se incorporation into the total platelet mass was calculated as previously described (7).

Student's *t* test was used to evaluate the results.

Results. Figure 1 shows the results of comparing $Na_2\,^{35}SO_4$ and ^{75}SeM as platelet labels for measuring the amount of stimulation of thrombocytopoiesis in mice after injection of various doses of TSF-rich material. The two isotopes showed similar responses, i.e., as compared to saline-treated controls, TSF caused significantly more isotope incorporation into platelets of both normal and RAMPS-treated mice at all TSF dose levels. Isotope incorporation into platelets increased as the TSF doses were increased, indicating

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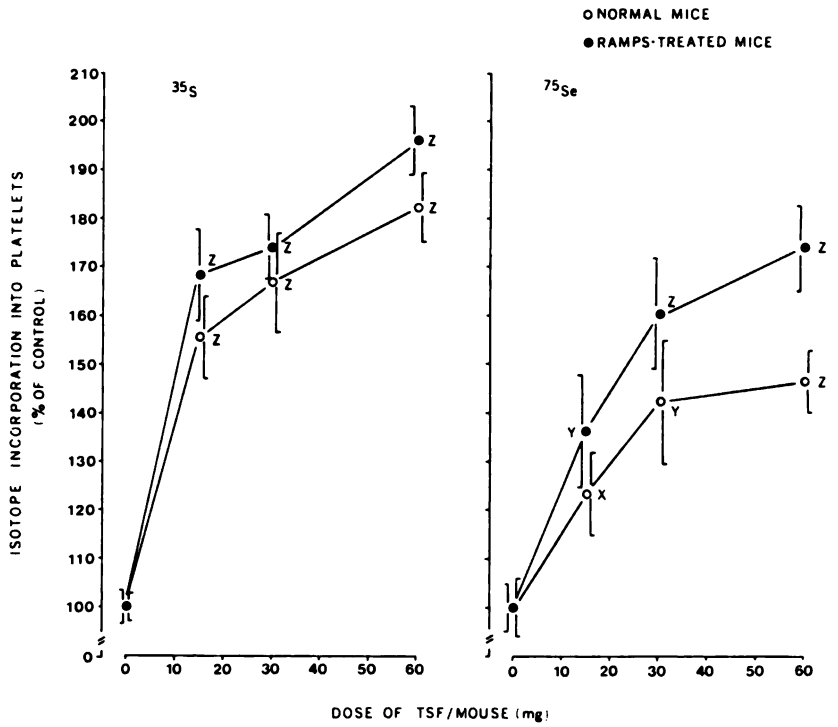

FIG. 1. Isotope incorporation into platelets of mice after injection with various doses of thrombopoietin (TSF)-rich kidney cell culture medium. Each point represents the average 8–35 mice and the vertical lines indicate the standard errors. ^{35}S ($Na_2{}^{35}SO_4$) was injected at 30 μCi/mouse and ^{75}Se ([^{75}Se]selenomethionine) was used at 1 μCi/mouse. The % isotope incorporation into platelets was determined 24 hr later using blood obtained by cardiac puncture. Values were significantly different from control: saline-treated control mice versus TSF-injected mice: (*X*) $P < 0.05$, (*Y*) $P < 0.005$, (*Z*) $P < 0.0005$.

that all four groups of animals had positive dose–response curves.

As already noted, RAMPS-treated mice, because of lower baselines, showed greater responses than did normal mice. Although not shown, RAMPS pretreatment reduced the isotope incorporation into platelets of saline-treated mice: % ^{35}S incorporation into platelets of 35 normal mice was $4.26 \pm 0.13 \times 10^{-3}$ compared to $3.26 \pm 0.01 \times 10^{-3}$ for 29 RAMPS-treated mice; for ^{75}SeM the % incorporation into platelets of 30 normal mice was $5.16 \pm 0.31 \times 10^{-2}$ versus $4.03 \pm 0.23 \times 10^{-2}$ for 24 RAMPS-treated mice. As depicted in Fig. 1, the percentage incorporation of ^{35}S was greater at each TSF dose level than the % ^{75}Se incorporation, e.g., the difference between % ^{35}S incorporation and % ^{75}Se uptake was significant after injecting TSF at 60 mg/mouse for both normal mice ($P < 0.005$) and RAMPS-pretreated mice ($P < 0.05$).

Discussion. Several attempts have been made to find the optimum conditions for the assay of TSF (7–11). Mice in rebound thrombocytosis were more sensitive to exogenous TSF preparations than were normal mice. Also, if the first of four TSF injections was given on Day 5 after RAMPS treatment and the 24-hr %^{35}S incorporation determined on Day 8, greater ^{35}S incorporation values were found than at the other times tested (11). The route of administering TSF did not seem to be important since TSF injected s.c. or i.p. gave essentially the same result (9). Multiple injections were more effective than single injections (9). Isotope incorporation gave a more sensitive measurement of thrombocytopoiesis than platelet counting or platelet sizing (8). Additional work (10) revealed that the time of measurement of ^{35}S incorporation into platelets and selection of the mouse strain determined to a large degree the

sensitivity of the TSF assay. Sex of the mice did not appear to be important; similar responses were found in both male and female mice (10). Our results show herein that although ^{35}S gave significantly greater values at the higher TSF doses, either [^{35}S]sodium sulfate or ^{75}SeM can be used to measure platelet production rates in mice stimulated with thrombopoietin.

For optimum results, great care should be used in selecting the mouse strain. The mice would be pretreated with platelet-specific antisera to make them thrombocytopenic (7–11). Five days later, when mice are thrombocytotic, they should be injected with test substances in multiple doses over a 2-day period, and platelet production rates measured by administering either Na_2 $^{35}SO_4$ or ^{75}SeM; isotope incorporation into platelets should be measured 24 hr later (10).

Summary. Platelet production rates were measured in thrombopoietin (TSF) assay mice by use of both [^{35}S]sodium sulfate and [^{75}Se]selenomethionine (^{75}SeM) as platelet labels. Although significantly higher values were found using Na_2 $^{35}SO_4$ than ^{75}SeM, the incorporation patterns for both isotopes showed similarities in dose–response experiments.

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Calcium on the Monomer-Dimer Association of Calcium-Binding Protein in the Serum of Laying Hens (40687)

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ing hen provides a good model for calcium in a biological system be- he marked changes in calcium me- ıssociated with reproduction (1). A containing 25 mg of calcium in a)0 ml of plasma (2), can deposit a aining 1.5–2.0 g of calcium in ap- :ly 15 hr (1). Laying hens have long wn to have a higher level of blood ıan males or nonlayers (3). Virtually ; increase is due to an increase in ıdiffusible calcium (4). The nature lecules responsible for the binding ɔpen to question. Evidence for and ılloidal calcium (5, 6), albumin (7, itin (9–12), and high molecular ıosphoproteins (13), has been pre- ıe molecule appears to bind a large f calcium but with an average dis- constant of 10^{-1} to 10^{-3} (14, 15). ased calcium present in the serum :nized roosters, was found to be with two proteins, X_1 (a phospho- nd X_2 (a lipoprotein) which could ted by ultracentrifugation (15). A ıg electrophoretic component was gel filtration to have a molecular 183,000 (28). The electrophoretic ɔf this component decreased as the ıncentration increased (29). This uted to a decrease in net charge on ule. The molecule appeared to bind nount of calcium. Kuan and Clegg l 110 calcium-binding sites with an n constant of 1.66×10^6. Deeley *et* und a single phosphoprotein, vitel- ı the serum of estrogenized roosters. 'e molecule, of 480,000 M_r, was a nposed of two 240,000 M_r subunits ined by gel electrophoresis. Vitellogenin is apparently broken down by proteolysis in the yolk into phosvitin and α- and β-lipovitellin. Bergink and Wallace (17) have also identified a 200–250,000 M_r phosphoprotein in estrogen-treated roosters. It seems possible that the calcium-binding proteins X_1 and X_2 observed by Urist (15) were the dimer and monomer forms of vitellogenin, and that the electrophoretically separated component I (29, 30) was the monomer form of vitellogenin.

In the related *Xenopus* system (18) estrogenized males contain a similar plasma phosphoprotein vitellogenin of about 400,000 M_r, which is also a dimer of approximately 200,000 M_r subunits.

The present investigation (19) was undertaken to determine the relation between monomer–dimer association and calcium binding of the calcium-binding protein in the blood of hens and estrogenized roosters.

Materials and methods. Animals and blood collection. Regularly laying, 1- to 2-year-old, single-comb, white Leghorn hens, weighing about 2 kg were used. They were fed a standard laying ration and water *ad libitum* until the night before blood collection when food was removed to keep blood lipid content low. Roosters were injected intramuscularly with 20 mg/kg body wt of estradiol valerate (E. R. Squibb & Sons, Inc.).

For blood collection, the birds were anesthetized by intramuscular injection of chloral hydrate (1.4 ml of a 35% solution). Blood was withdrawn from the carotid artery. Plasma was separated from the blood cells by centrifugation at 2000 rpm in a refrigerated Sorvall RC-2 centrifuge for 30 min.

Lipid flotation and preparation of Fraction 4. Lipid flotation was performed on plasma using a modification of the method of DelGatto *et al.* (20). Plasma (40 ml) was added slowly with gentle mixing to 80 ml of an ice-cold solution containing 3.94 *M* NaBr, 0.045 *M* NaCl, and 3.2 m*M* $CaCl_2$. The solution was then centrifuged in a Spinco Type

address: Department of Molecular Biophys- ıchemistry, Yale University, New Haven,). This work was performed as part of a presented by C. L. Hermsdorf to The Penn- te University.

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50 rotor at 43,500 rpm at 18° for 24 hr. The contents of the tube were divided into four equal fractions. The contents of the bottom quarter of each tube were dialyzed against the standard buffer (0.10 *M* NaCl, 0.01 *M* Tris hydroxymethylaminomethane, 0.01 *M* $CaCl_2$, and 0.02% NaN_3, adjusted to pH 7.5 with HCl) and labeled as Fraction 4.

In a preliminary experiment, Fraction 4 was shown to contain approximately 80% of all calcium-binding activity, as determined by equilibrium dialysis. Therefore, all further work was performed on this fraction.

Gel chromatography. Fraction 4 was purified on Sephadex G-200 (Pharmacia) and Sepharose gels 6B and 2B (Pharmacia) using 2.5 × 30 and 2.5 × 80 cm chromatographic columns (Glenco). Elution was by upward flow at rates not exceeding 0.5 ml/min. Ultraviolet absorption of the eluate was monitored at 254 nm with an Isco monitor and the absorption of the fractions after elution measured at 280 and 260 nm with a Beckman DU-2 spectrophotometer.

Protein concentrations were determined by the method of Lowry *et al.* (21) as modified by Oyama and Eagle (22). Carbohydrate was detected by the orcinol–sulfuric acid method of Francois *et al.* (23).

Equilibrium dialysis. Protein in standard buffer was placed on one side of the membrane of an equilibrium dialysis cell (Chemical Rubber Co.). Buffer plus $^{45}CaCl_2$ (New England Nuclear, carrier free) was placed on the other side. The solutions were maintained at 4° on a rotary shaker. The 25-μl samples were removed periodically (in triplicate) and counted on a Nuclear Chicago Model 1105, spectro-shield, low-background, gas-flow, planchet counter.

Analytical ultracentrifugation. Analytical ultracentrifugation was performed on the Beckman Model E analytical ultracentrifuge with schlieren and ultraviolet optics. Solution densities were determined by pycnometry. Viscosities were determined in Cannon viscometers. Boundary runs were performed in 12-mm Kel-F centerpieces at 44,770 rpm (24). Meniscus depletion runs were performed in double-sector cells (24) at 9945 rpm for 24 hr after an initial overspeed using schlieren optics.

Sucrose density gradient centrifugation. Linear 5–20% sucrose gradients containing 0.01 *M* Tris buffer were prepared at 4° and centrifuged for 27.5 hr in an SW 25 rotor in a Beckman L2-65B centrifuge at 23,000 rpm.

Results. Analytical sedimentation and chromatography of Fraction 4. Analytical ultracentrifugation of Fraction 4 using schlieren optics showed three components with sedimentation coefficients of 3.4–4.4, 5.9–8.0, and 11.6–15.6 S. Representative uncorrected sedimentation coefficients are given in Table I. Density and viscosity correction factors were less than 1%.

Sephadex G-200 chromatography of Fraction 4 gave two UV-absorbing peaks (Fig. 1), the first peak eluting in the void volume. Rechromatography of the first-peak material

TABLE I. REPRESENTATIVE PLASMA AND RED BLOOD CELL (RBC) VOLUMES, AND SEDIMENTATION COEFFICIENTS OF FRACTION 4 ENTITIES

Bird	Plasma volume (ml)	RBC volume (ml)	Plasma: RBC ratio	Sedimentation coefficients[a] (Svedberg units, S)		
3	40	ND[b]	—	4.0	7.0	14.3
4	45	25	1.8	4.4	8.0	13.5
6	26	ND	—	3.4	6.0	11.8
9[c]	90	80	3.0	3.5	7.9	11.6
16	52	ND	ND	4.0	7.1	15.6

[a] Uncorrected.
[b] Not determined.
[c] This was a long-term estrogenized bird.

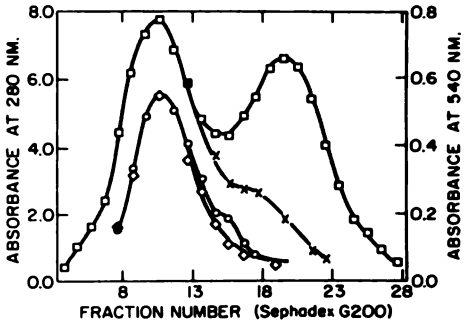
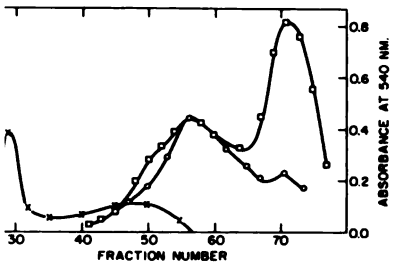


FIG. 1. Sephadex G-200 elutions of Fraction 4 (bird 9). UV absorption and carbohydrate profiles. The material from tubes 8–13 was combined, concentrated, and reeluted on Sephadex G-200. Carbohydrate (540 nm) was detected by the orcinol–sulfuric acid test. (□) Original Sephadex G-200 elution at 280 nm; (○) Sephadex G-200 reelution of material from tubes 8–13 at 280 nm; (×) original Sephadex G-200 elution at 540 nm; (◇) Sephadex G-200 elution of material from tubes 8–13 at 540 nm.



Sepharose 6B elution of Fraction 4 (bird 16). ırbohydrate profiles. The sharp dextran 2000 ɔe 29 denotes the void volume. Carbohydrate was determined by the orcinol–sulfuric acid 'alues from representative fractions: Tube 50: tube 56; 7.9, 16.4 S; tube 61: 7.8, 16.3 S; tube (×) Dextran 2000 at 254 nm; (□) absorbance (○) absorbance at 540 nm.

ılly removed trailing second-peak (Fig. 1). The elution profiles on x G-200 were similar for Fraction 4 ısma or serum, normal laying or es-ɛd birds. However, long-term estro-birds had a larger proportion of the k material. A bimodal elution profile ılted when Fraction 4 was chromat-ɜ on Sepharose 6B (Fig. 2). The dex-)0 elution pattern indicates that all 4 components eluted well after the ume. When material from the first ted on Sepharose G-200 (Fig. 1, tubes ıs chromatographed on Sepharose 2B : absorption peak was detected (data /n).

the peak materials from these col-ere tested by analytical sedimenta-first peak on both columns (Fig. 1, 11, and Fig. 2, Fraction 56) con-ıe two fast-sedimenting components 6 S); the second-peak material (Fig. on 20, and Fig. 2, Fraction 70) con-he slow-sedimenting component (4

arbohydrate content of Fraction 4 eluted on Sephadex G-200 and se 6B is shown in Figs. 1–2. In each first peak eluted, which contains the binding material of Fraction 4, con-bout 5% carbohydrate.

'zation of calcium-binding activity. ercent of the soluble calcium-binding in serum was found in the bottom (Fraction 4) of the tube following . Calcium binding was further local-ized by adding ^{45}Ca to Fraction 4 prior to sucrose density centrifugation. The UV absorption and ^{45}Ca profiles obtained following sucrose density gradient centrifugation of Fraction 4 indicated a peak of ^{45}Ca associated with the fast-sedimenting proteins (Fig. 3). The contents of tubes 9–11 (Fig. 3) were incubated with additional ^{45}Ca and eluted on Sephadex G-200; the protein eluted in the void volume and had ^{45}Ca associated with it. Materials from tubes 22–24 (Fig. 3), similarly treated, eluted later on Sephadex G-200 with no associated ^{45}Ca. Equilibrium dialysis confirmed that the fast-sedimenting, G-200 void volume material contained the calcium binder: This material bound 23% of the total calcium when the free calcium concentration was 1 m*M* and the protein concentration was 1.5 mg/ml.

Molecular weight determination. Resolution of the two fast-sedimenting entities by gel chromatography or sucrose gradient sedimentation was unsuccessful. The relative amounts of these two fast-sedimenting entities (8 and 16 S) were variable, and on several occasions only the fast-sedimenting entity (16

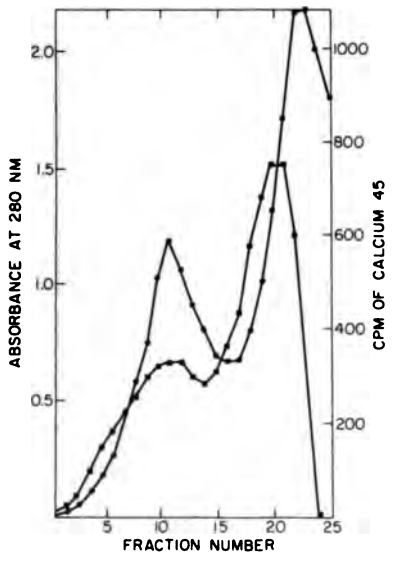


FIG. 3. Sucrose gradient UV absorption and ^{45}Ca profiles. Fraction 4 (bird 4) was layered on a 5–20% sucrose gradient and centrifuged at 4° at 23,000 rpm in an SW 25 rotor for 27.5 hr. Each fraction of the gradient was monitored for absorption at 280 nm and for ^{45}Ca. (In the absence of protein, ^{45}Ca decreased exponentially from the top of the tube). (■) Absorbance at 280 nm; (●) ^{45}Ca cpm.

S) was observed. When equilbrium sedimentation (meniscus depletion) was performed on material eluted first from a Sepharose 2B column a 5.4 ± 0.1 × 10^5 M_r was obtained (assuming $\bar{v}$ = 0.72) with no evidence of heterogeneity. After dialysis against 0.10 *M* citrate, 0.01 *M* Tris (pH 7.5), a 2.7 × 10^5 M_r was calculated. When material containing both peaks (16, 8 S) was subjected to equilibrium sedimentation, heterogeneity in molecular weight was apparent. However, when such fractions were first dialyzed against citrate, and then subjected to equilibrium sedimentation, molecular weights were similar to those obtained when the 16 S entity had been citrate treated. Analytical sedimentation confirmed that dialysis against citrate or EDTA converted a mixture of 8 and 16 S components to 8 S, and, furthermore, showed that subsequent dialysis of the 8 S component against calcium ions again produced a mixture of 8 and 16 S (Fig. 4).

Determination of frictional factor and axial ratio. The molecular weight and $S_{20,w}$ values obtained for the calcium-binding material were used to determine the molecular frictional coefficients and axial ratios, assuming a hydration of 0.2 g H_2O/g protein for the calcium-binding material (25). For the 5.4 × 10^5 M_r material a value of f/f_{min} of 1.6 and an axial ratio of 8.7 were determined. For the 2.7 × 10^5 M_r material, f/f_{min} was 2.0 and the axial ratio 16.0, assuming a prolate ellipsoid model.

Discussion. We have identified the major calcium-binding protein in chicken serum as an asymmetric (f/f_m = 1.6) 5.4 × 10^5 M_r dimer which dissociates in the absence of calcium into 2.7 × 10^5 M_r monomers which are also highly asymmetric (f/f_m = 2.0). The

FIG. 4. Analytical boundary sedimentation of "fast" calcium-binding material. The fast calcium-binding component isolated from a sucrose gradient (Fig. 3, Fractions 8–12) was treated as follows below and then spun at 44,770 rpm in an analytical centrifuge using schlieren optics. Frames are shown at 8-min intervals. (A) Sucrose gradient fractions 8–12 were pooled and dialyzed against Sephadex buffer containing 1 m*M* $CaCl_2$. The two sedimenting peaks have values of 17.4 and 10.4 S. (B) The pooled fractions (A) were dialyzed against 0.1 *M* EDTA, pH 7.5. This gave a value of 7.0 S. (C) The pooled fractions were dialyzed against 0.1 *M* sodium citrate, 0.01*M* Tris, pH. 7.5. This gave a value of 7.3 S. (D) The citrate was dialyzed out against sephadex buffer. Peaks of 16.6 and 9.6 S resulted.

partially reassociate to the dimer
:nce of calcium ions.
;phoprotein complex vitellogenin
:d by Deeley (16) in etrogenized
also a dimer of approximate
r consisting of lipovitellin and
The 450,000 M_r vitellogenin in the
:strogenized male *Xenopus* (18) is
/mmetric (f/f_m = 1.5; calculated
ata of Wallace (26)) dimer. The
nclusion from these comparisons
5.4 × 10^5 M_r calcium-binding
hicken serum is vitellogenin.
: sedimenting entities observed in
:entrifugation of the high-density
:tion may be identified as the di-
onomer vitellogenin and chicken
min. The two peaks eluting from
s are vitellogenin and serum al-
: lack of resolution of the mono-
ner forms by gel chromatography
ained by the extreme asymmetry
olecules. Recently Guyer *et al.*[2]
ı by gel chromatography the pres-
n serum of two calcium-binding
ıich they have identified immu-
as vitellogenin and chicken serum
hey find an estimated molecular
the calcium-binding protein re-
:llogenin of 6 × 10^5 M_r.
um-binding property of the vitel-
be attributed to both the phosvi-
ents which contain a high propor-
osphoserine and the phosphate
ent in the lipovitellin components
ıd by equilibrium dialysis that at
tion of 1.5 mg/ml protein and 1.3
m, 23% of the calcium is bound,
ı minimum of 100 calcium-bind-
the 5.4 × 10^5 M_r complex. Kuan
(30) found 110 calcium-binding
)lecule for their component I.
:rsible association–dissociation of
enin dimer–monomer system de-
he presence of calcium, and the
planation is that calcium bridges
:en two monomers. Dimerization
asymmetry slightly, suggesting
: association of rod-shaped mole-
arrangement of phosvitin and li-

ateful to Guyer *et al.* for a preprint of their
bmitted to Proc. Soc. Exp. Biol. Med.

povitellin in the dimer must be such that the phosphate groups are not available for further intermolecular association since larger complexes are not found. Attempts to observe the molecules directly by electron microscopy are in progress.

Summary. A major calcium-binding protein of laying hen plasma was fractionated and partially characterized. After lipid flotation, approximately 80% of the total binding activity was localized in a high-density plasma fraction. Chromatography on Sephadex G-200 or Sepharose 6B gave a bimodal elution pattern. Sucrose gradient centrifugation yielded a similar bimodal pattern; the fast- and slow-sedimenting ultraviolet (UV) absorption peaks appeared identical to the first and second chromatographic elution peaks, respectively. Equilibrium dialysis localized the calcium-binding activity to the faster-sedimenting material. An orcinol–sulfuric acid test indicated a total carbohydrate content of 5% for the fast peak.

Analytical velocity ultracentrifugation measurements of this calcium-binding component yielded varying amounts of two entities with sedimentation coefficients ($S_{20,w}$) of approximately 16 and 8 S. The 16 S material was shown by equilbrium centrifugation (meniscus depletion) to have a 5.4 × 10^5 M_r. Dialysis of the 16 S moiety against citrate or ethylene diamine tetraacetic acid (EDTA) buffer produced 8S material having a 2.7 × 10^5 M_r and this monomer–dimer association was partially reversible by subsequent dialysis against calcium ions. The calcium-binding molecule appears to be vitellogenin. Calcium-binding protein was found at elevated levels in plasma from long-term estrogenized birds but not from nonlaying birds.

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ıantitative Aspects of Lipoprotein Lipase Release by Heparin in Mice (40688)[1]

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ough heparin has been known to re- ipoprotein lipase (diacylglycerol acyl- ase, EC 3.1.1.34) since 1943 (1), the tative aspects related to dosage have been fully determined. Furthermore, ıe data of Robinson *et al.* (2) are avail- ɔncerning the half-life of enzyme ac- nce released into the blood. Olsson *et* and Estes *et al.* (4) published data on lume of distribution of heparin and data on heparin's anticoagulant action ı. More recently, McAvoy (5) deter- the biologic half-life of heparin. In this we present quantitative dose–response n the release of lipoprotein lipase by n in mice, as well as information on uration action of released lipoprotein in serum.

erials and Methods. Seventy male Webster mice (weighing 20–30 g) were n this investigation. Heparin sodium jected intravenously in the tail vein in me of 0.1 ml. Axillary blood was ob- from etherized mice, allowed to clot, en spun down in a refrigerated centri- t 2500 rpm for 30 min at 5°. (In pre- :xperiments we found that etherization t affect lipoprotein lipase release.) The erum was either tested immediately or at −10° and used on subsequent days. ızyme was found to be quite stable and d no discernible diminution in activity :age at −10° for 20 days. It was always either immediately or within a few fter collection.

protein lipase assay. To determine the ɔf lipoprotein lipase, 0.6 ml of serum dded to a flask in which 5.4 ml of iolein substrate (see below) was placed; incubated in a shaking incubator for at 27° (according to a modification of :thod of Schotz (6, 7)). At intervals of), and 20 min, 0.5-ml samples were removed from the flask and added to isopropanol–sulfuric acid (40:1) solution to stop the reaction. Then 5 ml of hexane and 1 ml of water were added to each aliquot and the tubes were shaken for 1 min in a mechanical shaker. The upper layer containing the hexane phase was separated and shaken with 2 ml of 0.1 *N* KOH for 10 min. The lower phase contains the potassium oleate generated by enzymic action on the triolein substrate. The 1-ml aliquots of the lower phase were added to 10 ml Aquasol and counted in an Intertechnique liquid scintillation spectrometer, Model SL-30. All results were corrected to dpm and converted to nmol oleic acid generated/min.

Triolein substrate for enzyme assay. The 3 ml of ^{14}COOH-triolein (New England Nuclear Corp, 55 mC/m*M*) in hexane solution and containing 15 μCi of radioactivity was evaporated to dryness under nitrogen and 270 mg triolein (fatty acid free), was added to the beaker. Fatty acid-free albumin (600 mg) was added and 1.8 ml Triton X-100 (1%) and 34.2 ml Tris buffer (0.2 *M*, pH 8.0). The mixture was sonicated in an ice bath for 5 min at full output of a Bronson sonicator. The emulsion was stable for at least 30 min. If not used within 30 min, the emulsion was resonicated before use. The emulsion could be stored by freezing at −10°, thawed, and resonicated before use.

All results are expressed as the mean ± SE. Triolein was 99% pure and obtained from Sigma Chemical Company as well as fatty acid-free albumin (from fraction V). Triolein-^{14}COOH was obtained from New England Nuclear and has a specific activity of 55 mCi/mmol. Heparin sodium was made from porcine intestinal mucosa and was obtained from Sigma Chemical Company. It was assayed by the company at 169.9 U.S.P. units/mg.

Results. Control studies in fasted animals in which saline was injected intravenously, showed extremely low levels of nonspecific

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lipase activity that generated less than 0.01 nmol oleic acid/min. This amount of enzyme activity was negligible compared to the quantity of oleic acid generated when heparin was given. Control substrates without enzyme showed a small degree of hydrolysis on incubation and produced the equivalent of 0.5 nmol oleic acid. This value was subtracted from the results reported and was consistent in all experiments.

To determine levels of serum lipoprotein lipase, groups of five mice were injected with heparin, i.v., at six dosage levels of 0.1, 0.5, 1.0, 4.0, 8.0, and 12.0 units/g body wt. The animals were bled 8 min after the injection and the serum prepared as indicated under Methods.

The results of this study show that with doses of 0.1 unit/g there are small but measureable levels of lipoprotein lipase released into the blood (Fig. 1). With increasing dosage of heparin above 0.1 unit/g, there was a consistent and proportional increase in the amount of lipoprotein lipase released into the blood. The quantity of enzyme released into the blood appeared to reach a plateau maximum at a dosage of heparin of 4 units/g. The form of the dose–response curve for the release of the enzyme into blood was sigmoidal and typical of classical dose–response curves. It is shown in Fig. 1.

In additional experiments in five mice, a dose of heparin of 0.05 units/g, in some animals caused the release of lipoprotein lipase activity into the blood and in others was almost without effect. These actions were not studied further because of great variability and therefore are not included in the graph shown in Fig. 1. This dose is probably threshold in a number of mice.

Following these experiments, we studied the time-duration characteristics of released lipoprotein lipase in blood. We employed five groups of seven mice and injected each mouse with 1 unit/g heparin, i.v. Blood was obtained from these mice at 30 sec, and 1, 5, 15, and 60 min following the injections. The sera were prepared as indicated before, and the levels of lipoprotein lipase were determined.

The data (Fig. 2) showed that heparin released lipoprotein lipase rapidly on entering the vascular system. The earliest practical time for measurement was 30 sec after the administration of heparin. Such measure-

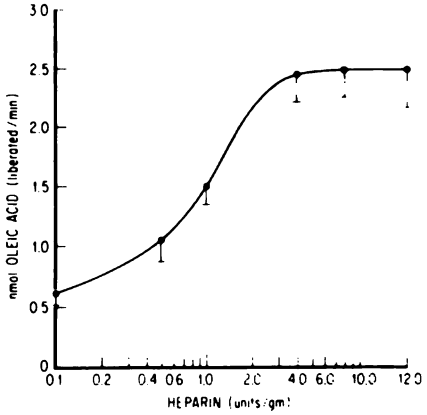

FIG. 1. Curve of levels of lipoprotein lipase present in serum 8 min following the intravenous injections of graded doses of heparin. Activity of lipoprotein lipase is given as nmol oleic acid liberated/min by enzymic action on [^{14}C]triolein substrate (see text).

ments were extremely difficult to do accurately and showed very high levels of the enzyme in serum. However, due to very high errors in sampling, these data are not included in Fig. 2.

Once released into the blood, there was an extraordinary rapid decay of lipoprotein lipase levels in serum. Graphical estimation of the half-life, showed a half-life of lipoprotein lipase in serum of approximately 4 min. As indicated in Fig. 2, at 1 min after injection, serum lipoprotein lipase was at the highest level in these experiments and showed activity equivalent to 3.8 nmol oleic acid liberated/min. At 5 min following injection the enzyme activity in the serum had dropped to 1.8 n*M* oleic acid liberated/min. Sixty minutes after injection the serum lipoprotein lipase levels had returned almost to control values. Three hours after injection there was no discernible activity in the serum. Thus, it appeared from these studies that the total effective period for lipoprotein lipase activity in the serum, once released by heparin (1 unit/g), was at most 1 hr following injection.

To obtain some preliminary understanding of the disposition of serum lipoprotein lipase once released into the blood, mouse liver homogenate in isotonic saline was prepared and incubated in duplicate with serum (0.5 ml) at 37° containing known levels of the enzyme. Thirty minutes after incubation samples were taken and measured for lipoprotein lipase activity. After correcting for dilution,

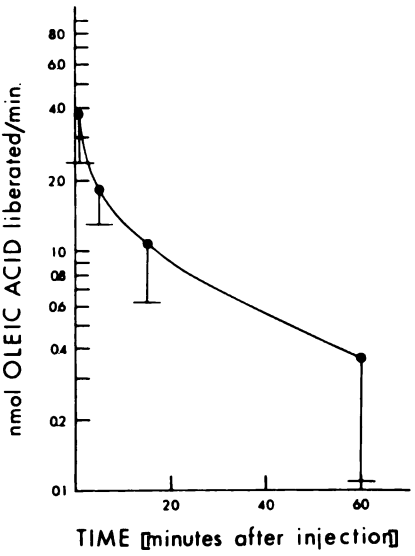

FIG. 2. Disappearance of lipoprotein lipase activity m serum following its release by the intravenous ections of 1 unit/g heparin at zero time. Activity of : enzyme is given as nmol oleic acid liberated/min by ymic action on [^{14}C]triolein substrate (see text).

: results of two experiments showed that out 40% of the lipoprotein lipase activity :sent in the original serum disappeared er 30 min.

Discussion. These data show that the re-ıse of lipoprotein lipase in mice follows a ıssical sigmoidal dose–response curve. Fur-:rmore, the effective range of this dose-ıponse curve is from 0.1 to 12 units/g of parin. It is evident from the curve gener-:d that no greater increase in lipoprotein ıase activity occurred with doses higher an 4 units/g heparin. Even though heparin s been known to release lipoprotein lipase r many years, exact dose–response data ve been lacking. Other than the report of)binson and Harris in man, there have not en quantitative data of this kind.

The results on the half-life of free lipopro-n lipase in serum were similar in some ıpects to man, where a longer half-life of 9 n has been reported by Robinson and Har-. There is no doubt that in mice, once the zyme is released from its storage sites, it dergoes rapid disappearance from the)od. Our preliminary results with liver ho-)genate reveal only that liver can inactivate : enzyme molecule. More sophisticated experiments would have to be done to determine whether this is outright destruction of the enzyme or merely complexing with charged anionic compounds.

Although, *in vivo*, in the clearing of chylomicrons from blood, there is evidence that lipoprotein lipase remains attached to the capillary endothelium (8), once the enzyme is released free into the circulating blood by heparin treatment, enzyme activity diminishes rapidly. This suggests that the functional groups necessary for lipolytic activity are very quickly blocked or destroyed.

In man, lipoprotein lipase once released has a comparable short half-life to that observed in mice in these experiments. Unless there is careful consideration of the time interval following heparin injection, erroneous interpretations will result concerning the levels of serum lipoprotein lipase. It can be seen from Fig. 2, that had we chosen 1 min following injection for our dose–response curve in Fig. 1, the levels of lipoprotein lipase would have been almost threefold higher.

There have been no studies to date, to indicate how rapidly lipoprotein lipase is synthesized to replace that which is released by heparin or other polyanionic drugs. It is unlikely to be related to the half-life reported for heparin (4, 5). Such data would be extremely valuable for determining the kinetics and turnover rate of this enzyme, particularly because so many individuals are being treated with heparin.

We wish to thank Paul Segal and Martin Ontell for their technical help in this project.

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Pain Perception in Drug-Free and in Methadone-Maintained Human Ex-Addicts (40689)

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It has been asserted that narcotic addicts are more sensitive to pain (1) and less able to tolerate discomfort (2) than nonaddicts. On the other hand, concern has been expressed that the perception of pain by methadone-maintained ex-addicts might be impaired by a supposed analgesic effect of their daily narcotic medication (3). The study reported here investigated the pain threshold and pain tolerance of ex-addicts with long histories of heroin addiction, and of age-matched, never-addicted siblings of addicts. Resting blood pressure was determined before and after testing to further explore the changed autonomic responsivity found in the early postaddict state (4, 5).

Materials and methods. Subjects and drugs. Thirty men and women served as *S*s in three agematched groups of 10 each: drug-free ex-addicts (DFE), methadone-maintained ex-addicts (MME), and nonaddict siblings (NAS). Paid volunteers were recruited from a group of potential *S*s who had already completed a Cornell Medical Index and a 1-hr drug and life-history interview. This larger subject pool included ex-addicts contacted through abstinence drug treatment programs and methadone maintenance clinics in New York City and their nonaddict siblings whenever possible. *S*s were selected so that there were no significant differences between groups in age, ethnic origin, sex, or work status. Altogether there were 24 men and six women; 16 *S*s were black, 6 were Spanish, and 8 were white. Relevant aspects of the drug history of MME and DFE are shown in Table I. The two ex-addict groups were comparable in addiction history and in length of either methadone maintenance or abstinence treatment. One-way analyses of variance were used to examine group differences in the subject characteristics summarized in Table II. *S*s in both MME and DFE reported more medical problems and had completed fewer years of school than did those in NAS. Analyses of urine specimens collected just before the individual testing sessions were negative for amphetamines, barbiturates, and narcotics in DFE and NAS and were positive for methadone only in MME (6). The mean daily methadone dose in MME was 80 mg, with a range of 60–100 mg, and with a mean of 18 months since any dose change.

Procedure. The procedure was described in detail to the *S* before the testing began and a written consent was obtained with the understanding that the *S* could withdraw from the experiment at any time. All *S*s completed the experiment. All tests were conducted in the afternoon in an air-conditioned room. The *E* avoided systematic knowledge of the status of each *S*, although occasionally there were visible scars on the arm or a spontaneous remark of the *S* revealed group status.

Immersion of the hand in an ice-water bath was the stimulus used to produce what has been described as "deep aching pain" (7), the intensity of which has been shown to increase with the time the hand is immersed (8). The *S* was asked to report when "the feeling of cold turns into a feeling of pain" (threshold latency measured by *E* in seconds) and to remove the hand from the ice water when "it becomes too uncomfortable to continue" (tolerance measured in seconds). The trial was ended by *E* after 60 sec if *S* had not already said "stop" and withdrawn the hand since cold-produced pain intensity reaches its maximum at about 1 min and subsides thereafter (9). (The eight *S*s for whom this was necessary were distributed among the three groups: MME, 2; DFE, 2: NAS, 4). During the tests, the *S* sat on a swivel chair between two water baths that could be reached comfortably with either hand. Each trial began with a 2-min immersion of the left hand in a 30° adapting bath. The *S* quickly dried that hand and plunged it to the wrist in ice water (1°). This procedure was alternated between hands for a total of six trials, three with each hand, over a period of about 20 min.

Blood pressure was recorded with the *S* in

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E I. DRUG HISTORY OF MME AND DFE[a]

	MME	DFE
:s addicted	9.5 ± 2.1	9.0 ± 1.9
vely addicted	6.9 ± 1.1	5.2 ± 1.0
:hadone maintained	3.1 ± 0.6	—
g free	—	3.7 ± 0.7

; actively addicted represents time addicted to
1 the street" excluding time spent in institutions
on the street." Values are expressed as means

ıe position at 5-min intervals three
efore and three times after the series
tests.

treatment. The first pain test with
nd was considered a practice trial and
carded. Since the distributions of both
ıreshold and pain tolerance were
, log transformations of the data were
)efore three-way (groups × hand ×
ıalyses of variance with repeated mea-
'ere performed (10). Resting systolic
ıstolic blood pressure measurements
lso separately subjected to three-way
× before-after × order-of-measure-
ınalyses of variance.

lts. The pain threshold of the left hand
ghtly but significantly lower than that
ight hand (see Table III) as had been
d for a different subject population
). Tolerance was also slightly lower in
hand but this effect was not significant
).05 level. There were no other signif-
nain effects or interactions between
, hands, or trials.

:e separate statistical comparisons were
o test the specific hypotheses of pain
tion in narcotics addicts mentioned
To examine the directional hypothesis
:ed by Knowles *et al.* (1) that exaddicts
)re sensitive to pain than nonaddicts,
oled scores of DFE and MME were
red to those of NAS. This comparison
l an $F(1, 27) = 3.61$, $P < 0.05$ (one
, indicating that the ex-addicts had a
:antly lower pain threshold than the
dicts. A similar comparison failed to
t the claim of Martin and Inglis (2)
ain tolerance is lower in ex-addicts.
:rmore, an orthogonal comparison of
o ex-addict groups showed no signifi-
ifference in either threshold or toler-
These data do not support the conten-

tion of Lennard *et al.* (3) that stable daily doses of methadone "reduce perception" or "dampen feeling."

Nonaddict subjects had significantly lower resting diastolic blood pressures than the two ex-addict groups (see Table IV). Systolic pressures were also lower but the F obtained fell just outside the 0.05 region of rejection. There is a significant group × before–after interaction indicating that after the testing session, diastolic pressures were higher in the nonaddicts but not in the ex-addicts. There were no significant elevations of systolic pressure in any group following the ice-water stimulus.

Discussion. The absence of a significant difference in either the threshold or tolerance to cold-induced pain between the two ex-addict groups is of interest as it relates to the endorphin–opiate receptor systems. In subjects with a 60–100 mg/day intake of methadone, and an average concentration in plasma of about 3×10^{-7} M (13), an appreciable fraction of the endogenous ligand must be chronically displaced from the opiate receptors. The pain perception test used in this study should have been sensitive enough to reveal even a small difference between the two groups, if one existed, since it did show a significant difference between the hands of the same subject, and between the combined scores of the ex-addict groups and the nonaddict sibling controls. The similarity in response to cold-induced pain in two groups with different ligands associated with their opiate receptors suggests either that this receptor system does not affect the response to experimentally induced pain (14) or that methadone in the stabilized patient serves as a functional equivalent of the natural ligand. Indeed, two studies on human pain percep-

TABLE II. AGE, EDUCATION, AND SYMPTOM REPORTS[a]

	MME	DFE	NAS
Years of age	30.7 ±2.2	31.2 ±1.9	31.1 ±1.5
Years of education*	10.9 ±0.4	11.4 ±0.2	13.1 ±0.5
Positive responses to Cornell Medical Index**	21.9 ±4.2	26.9 ±4.9	8.5 ±1.8

[a] Values are expressed as mean ± SE.

* $F(2,25) = 8.12$, $P < 0.001$; total $n = 28$ due to missing data.

** $F(2,27) = 4.51$, $P < 0.05$.

TABLE III. PAIN THRESHOLD AND PAIN TOLERANCE[a]

	Threshold		Tolerance	
	Left hand*	Right hand	Left hand**	Right hand
MME	1.1852 ± 0.0691 (15.3 sec)	1.2197 ± 0.0808 (16.6 sec)	1.4090 ± 0.0592 (25.6 sec)	1.4539 ± 0.0641 (28.4 sec)
DFE	1.1001 ± 0.0531 (12.6 sec)	1.1878 ± 0.0501 (15.4 sec)	1.4133 ± 0.0589 (25.9 sec)	1.4743 ± 0.0437 (29.8 sec)
NAS	1.2838 ± 0.0440 (19.2 sec)	1.3318 ± 0.0579 (21.5 sec)	1.5104 ± 0.0546 (32.4 sec)	1.5029 ± 0.0552 (31.8 sec)

[a] Values are expressed as the mean ± SE of the log transform of two determinations with each hand. Values in parentheses are geometric means (antilogs of the means of transformed data) expressing the central tendency in seconds.

* Left-hand < right-hand threshold $F(1,27) = 9.11$, $P < 0.01$.

** Left-hand < right-hand tolerance $F(1,27) = 4.09$, $P < 0.10$.

TABLE IV. RESTING SYSTOLIC AND DIASTOLIC BLOOD PRESSURE[a]

	Systolic* (mm Hg)		Diastolic** (mm Hg)	
	Before	After	Before	After
MME	112 ± 4	112 ± 4	80 ± 4	78 ± 2
DFE	122 ± 6	122 ± 6	81 ± 4	82 ± 4
NAS	106 ± 4	107 ± 4	64 ± 2	71 ± 4***

[a] Values are expressed as means ± SE of the three measures made before and the three made after the pain-testing session.

* Group $F(2,27) = 3.22$, $P < 0.10$.

** Group $F(2,27) = 5.18$, $P < 0.05$.

*** Group × before–after interaction $F(2,27) = 3.48$, $P < 0.05$.

tion following naloxone have failed to demonstrate the involvement of endorphins in experimental pain of nonaddicts (15, 16). In practical terms, the important finding is that the perception of somatic pain is not impaired in the methadone patients, despite the daily ingestion of a medication that would be a powerful analgesic for nontolerant subjects.

Prystav (4) found short-term drug-free addicts to show no rise in diastolic blood pressure following various experimental presentations including cold pressor stimulation, while the pressure of control *Ss* rose. The results reported here indicate a similar lack of blood pressure response in long-term ex-addicts, both drug free and methadone maintained, when compared to a matched nonaddict group. The differing experimental procedures of the Prystav study (including presentations of white noise, strobe light, cold pressor stimulation of the left foot for several standard 1-min exposures, lack of pain perception tasks) make the parallel finding the more interesting.

The findings of this study indicate a differing physiological response to cold-produced pain in ex-addicts both methadone maintained and drug free compared to closely matched nonaddicts. Since the preaddiction pain sensitivity and blood pressure response of the ex-addicts are not known, there is no way to determine the origin of these differences, whether they predated or were the result of narcotic addiction. These different responses to a pain stimulus were found after prolonged abstinence in the case of one ex-addict group (DFE *Ss* were drug free more than 3 years) and suggest a chronic physiological difference. Finally, of perhaps greatest clinical importance is the finding that in stablized methadone-maintained ex-addicts (MME *Ss*' methadone dosage had not recently been raised or lowered) responses to pain do not differ from matched drug-free ex-addicts.

Summary. Cold-induced pain threshold and pain tolerance were measured in drug-free ex-addicts (DFE), in methadone-maintained ex-addicts (MME), and in age-matched nonaddict siblings (NAS). Multiple responses to an ice-water stimulus were obtained for each hand. Resting systolic and diastolic blood pressures were determined across a 15-min period both before and immediately after the pain-testing session. Pain threshold of both ex-addict groups (DFE and MME) was lower than that of NAS. Pain tolerance was slightly but not significantly lower. Ex-addicts further differed from nonaddicts in an autonomic response to the experimental situation: The diastolic blood pressure of nonaddicts rose following cold pressor stimulation but there was no rise in

the ex-addict groups. Methadone-maintained ex-addicts do not differ from drug-free ex-addicts in pain threshold, pain tolerance, or blood pressure response.

We thank the directors, particularly Dr. Mitchell S. Rosenthal of Phoenix House, the staff, and patients of various drug-free programs and methadone clinics for their cooperation in contacting or serving as subjects. The study was supported by NIDA Grant DA-00390-01 and by New York State Office of Drug Abuse Services Grant C-107653.

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Immunoenzyme Assay for Human Prostatic Acid Phosphatase (40690)

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Determination of circulating acid phosphatase (EC 3.1.3.2) has been a useful procedure, primarily in the assessment of metastatic carcinoma of the prostate (1, 2). Since the demonstration of the distinct antigenic specificity of human prostatic acid phosphatase (PAP) by Shulman and his co-workers (3), several immunochemical assay methods for this enzyme have been introduced and found to be superior to the enzymatic activity assay method for the diagnosis of prostatic cancer (4–10). During the elaboration of a radioimmunoassay based on the Farr method (11), we realized that it is possible to measure the enzyme activity (as well as the radioactivity) of ^{125}I-labeled PAP in ammonium sulfate-precipitated antigen–antibody complexes. This approach is comparable to the histochemical demonstration of enzyme activity in the immune complexes in radial immunodiffusion (5) and immunoelectrophoresis (4, 9, 10). This modified Farr technique (11), which we call immunoenzyme assay (IEA), has an advantage over radioimmunoassays (6–8) in its simplicity and speed. Counterimmunoelectrophoresis (9, 10) is basically a qualitative method while IEA is quantitative. In a preliminary screening of clinical serum samples for the PAP determination, excellent correlation was demonstrated between IEA, double-antibody radioimmunoassay, and counterimmunoelectrophoresis.

Materials and methods. Chemicals. Saturated ammonium sulfate [$(NH_4)_2SO_4$] was prepared in double-distilled water as a stock solution and stored at 4° with crystals remaining in the flask to prevent the solution from becoming supersaturated. Disodium *p*-nitrophenylphosphate was obtained from Calbiochem (San Diego, Calif. 92112). All of the assay tubes (10 × 75 mm, glass) were siliconized to reduce the nonspecific binding of acid phosphatases. Up to 8% of the enzyme binds to the glass wall without siliconization. Siliclad (Clay Adams, Parsippany, N.J. 04054) was used for the siliconization of glass tubes according to the instructions of the manufacturer.

Serum specimens. Normal human sera were obtained from volunteers among the laboratory staff and medical students. Over 200 normal sera were collected from apparently healthy male and female volunteers. Some clinical serum specimens (70) which showed normal range of serum acid phosphatase values by routine spectrophotometric assay at clinical laboratories were obtained from the Departments of Obstetrics and Gynecology and Oncology. The sera of prostatic carcinoma patients (50 males; age 57–72, average age 56) were provided by the Department of Urology, Wayne State University–Detroit Medical Center. Sera were stored frozen at −20° until they were assayed. Those human materials were obtained with informed consent; the WSU Human Experimentation Committee approved this study.

Standard prostatic acid phosphatase. The prostatic acid phosphatase was purified from pooled human semen. Methods of purification and physicochemical characterization for this enzyme have been described previously (12). The PAP preparation used in this study was free from any extraneous proteins judged by immunochemical criteria, was homogeneous in terms of molecular weight (M_r 100,000), and had specific enzyme activity of 500 units/mg protein. One unit of enzyme catalyzed the hydrolysis of 1 μmol of *p*-nitrophenylphosphate per minute. Concentrations of purified enzyme solutions were determined by absorbance measurement at 280 nm, assuming $E^{1\%}_{1cm} = 14.4$ (13). Kinetic parameters of purified PAP have been described previously (12).

Anti-prostatic acid phosphatase sera (anti-

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ıration and the specificity of anti-
ıve been reported previously (14,
ı of rabbit anti-PAP serum, R14,
this study and the concentration
antibodies were estimated by
precipitin and radioimmunoas-
g antibody N/ml.

nzyme assay (IEA). For testing
ıa or serum specimens, standard
ıns were prepared in a batch of
nan female serum of which the
PAP was established by the ra-
assay method. Serial dilutions of
P were made to yield 250, 125,
5.6, and 7.8 ng PAP/ml. These
rd solutions were distributed in
ıssay tubes in a volume of 100 μl
d stored frozen until use. Tubes
00 μl of PAP-free female serum
e control.

ıti-PAP serum, R14, and a batch
ıized normal rabbit serum, NR6,
ł 10-fold in phosphate-buffered
), pH 7.2. To one of the triplicate
containing 100 μl of either stan-
ır clinical serum samples, 100 μl
NR6 was added. This series of
l as the control group. To two of
e assay tubes, 100 μl of diluted
ɛrum, R14, was added and this
es served as the assay group.

es were then incubated at 4° for
100 μl of cold 100% saturation of
solution (767 g/liter) was added
ɛ to precipitate the antigen–anti-
exes. After an additional 60-min
ıt 4°, the tubes were centrifuged
5 min at 1500*g* to sediment the
ibody complexes.

ıoving the supernatants, the pel-
ısolved in 100 μl of 0.1 *M* acetate
ffer, pH 5.0, at room temperature
ıixing. In most of the assays, the
ıved completely to yield a clear
ese dissolved immune complexes
ɛd for 2 min at 37° in a heating
ıter bath. The enzyme assay was
the addition of 50 μl of 40 m*M*
ylphosphate to each tube, and the
ɛtion was allowed to proceed for
37°. The enzyme reaction was
the addition of 0.7 ml of 0.2 *M*
s imperative to follow the rigid
time course for each assay tube to obtain reproducible accurate assay data. The resulting *p*-nitrophenol concentration was determined by the absorbance at 410 nm. A standard correlation curve was established by plotting the absorbance (410 nm) at each endpoint against the mass of PAP in each corresponding tube.

Other biochemical and immunological assay. Serum acid phosphatase of each sample was assayed at the clinical laboratory before they were referred to this laboratory. Clinical laboratories at Wayne State University–Detroit Medical Center adopted the automated procedure of Roy *et al.* (16) for the measurement of serum acid phosphatase. In this laboratory, each clinical sample was assayed for PAP by a double antibody radioimmunoassay and a counterimmunoelectrophoresis and the details of the assay procedures were reported previously (8, 10).

Results and discussion. In a previous investigation, we confirmed the immunologic specificity of the PAP-anti-PAP reaction (14, 15). Human acid phosphatases remain soluble at 33% saturation of $(NH_4)_2SO_4$ (15), whereas PAP-anti-PAP immune complexes are quantitatively precipitated at this concentration of $(NH_4)_2SO_4$ (Fig. 1). Coprecipitation of unbound PAP and of nonprostatic acid phosphatases at 33% saturation of $(NH_4)_2SO_4$ was 7–10%. The primary antigen–antibody interaction reaches nearly 80% of the equilibrium level within the first hour of incubation (Fig. 2). Therefore, a short incubation (1-hr) period was chosen for all of the following experiments. Other investigators (11, 17, 18) have demonstrated that antibody does not release, exchange, or bind appreciable amounts of antigen after the addition of $(NH_4)_2SO_4$. However, the concentrations of antibody are critical for the quantitative recovery of immune complexes in this technique (Fig. 3). Under the conditions of antibody excess, over 80% of antigen was recovered as immune complexes during 1-hr incubation at 4°. Therefore a 10-fold dilution of anti-PAP was used in the standard assay.

In the standard assay method, immune complexes are precipitated by 33% saturation of $(NH_4)_2SO_4$ and the amount of PAP in the complexes are estimated by the average velocity of enzymatic activity. In separate ex-

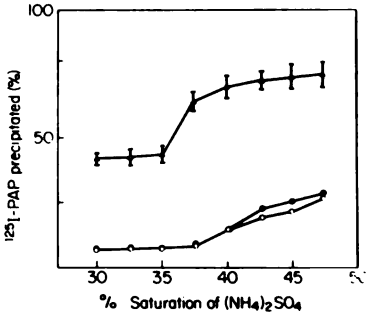

FIG. 1. Salting out of PAP by $(NH_4)_2SO_4$ in the presence of immunoglobulins. A series of tubes containing 100 μl of ^{125}I-labeled PAP (10 ng/ml) were incubated with equal volume of diluted rabbit sera (1:500), either anti-PAP serum, R14 (△), anti-human-serum-albumin serum (●), or normal serum, NR1 (○) for 1 hr at 4°. At the end of incubation, 100 μl of $(NH_4)_2SO_4$ was added to the final concentration of $(NH_4)_2SO_4$ of 1.33–1.9 *M* which was equivalent to the 30–47.5% saturation of $(NH_4)_2SO_4$. After centrifugation, the radioactivity of the pellet was counted and the amount of ^{125}I-labeled PAP precipitated was expressed as the fraction of the total ^{125}I-labeled PAP added. Each point with bars represents the average of five determinations and the ± 2 SD range.

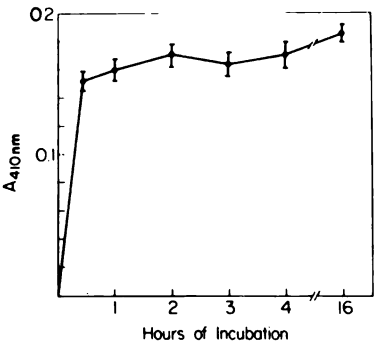

FIG. 2. The time course of the binding of PAP to anti-PAP antibodies. One hundred microliters of PAP (30 ng/ml) and anti-PAP serum, R14, (1:10) were incubated at 4° for various periods, the resulting antigen–antibody complexes were precipitated by $(NH_4)_2SO_4$, and the amounts of PAP in the complexes was estimated by enzyme assay. Each point with bars represents the average of five determinations and the ± 2 SD range.

periments, the catalytic activity of PAP was found to be unaffected either by $(NH_4)_2SO_4$ (up to 2.0 *M*) or by the presence of antibodies. The residual amounts of $(NH_4)_2SO_4$ carried through the precipitation and resuspension of antigen–antibody complexes may not affect the enzymatic activity of PAP. Figure 4 illustrates the correlation between the PAP concentration and its enzymatic activity. Twenty consecutive runs of standard PAP dilutions yielded linear correlation curves with identical slope and intercepts. The overall performance of this technique is shown in Table I.

Critical conditions in this assay are: (a) temperature, (b) substrate concentration, and (c) background enzymatic activity in the normal rabbit control tubes. All of the assay tubes were equilibrated to minimize the effects of temperature, and the time intervals for the addition and mixing of substrate were kept identical from tube to tube. Probably these steps would limit the handling of many

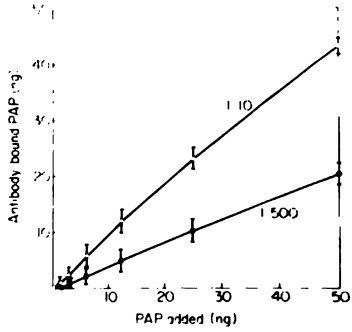

FIG. 3. Effect of anti-PAP antibody concentration in the PAP-anti-PAP interactions. To a series of tubes containing 100 μl of various concentrations of PAP (0–500 ng/ml), an equal volume of diluted (1:10 or 1:500) R14 serum was added, the immune complexes were precipitated by $(NH_4)_2SO_4$ at the end of incubation, and the enzyme activity of each pellet was determined. The masses of recovered PAP were calculated from the enzyme activity and the specific enzyme activity of the original PAP. Each point with bars represents the average of six determinations and the ± 2 SD range.

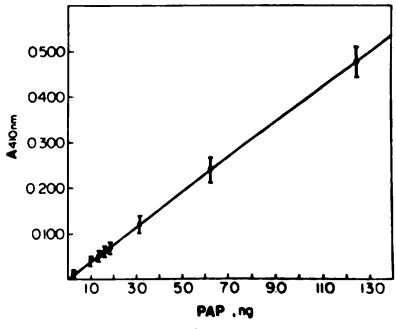

FIG. 4. Standard curve for IEA for PAP. Each point with bars represents the average of 20 independent determinations and the ± 2 SD range. The least squares slope is 0.0368. Details of the assay method are described under Materials and Methods.

BLE I. ASSAY PERFORMANCE OF IEA FOR PAP

ısitivity: Lowest detectable dose—4 ng/ml
producibility: Variation within repeated estimates in different assays

mple	No. of assays	PAP (ng/ml)[a]	CV (%)
L	20	23 ± 7.0	12.5
P	20	38 ± 4.5	4.1
G	20	124 ± 7.0	2.4

:uracy: Closeness of measurements to the true value

ple	Std. PAP added (ng PAP)	No. of assays	Recovery (%)[a]	CV (%)[b]
1	3	24	94 ± 11.8	4.5
1	6	24	98 ± 10.3	6.6

ean ± standard deviation (SD).
oefficient of variation (CV).

les at one time unless the system could ıtomated. As described in the previous rs (12, 14, 15), the K_m of PAP isozymes nitrophenylphosphate range from 4.1 to 10^{-4} M. Therefore, approximately 20-K_m was chosen as the substrate concen-n, and the initial velocities of the enzy- (0.5 to 25 ng) activity showed linearity · this substrate concentration.

e complication of this assay was a high ground enzymatic activity associated certain control serum samples. High ground enzymatic activity was found two types of specimens. Specimens with plasma protein concentration seemed to ise to slight turbidity after resuspension he immune complexes after the $_2SO_4$ incubation and this turbidity in-ed with the absorbance reading. Speci-containing a high concentration of acid hatases tend to give rise to a significant nt of residual acid phosphatase to the $_2SO_4$ precipitates of normal rabbit γ-lins and caused high absorbance read-· the control tubes. The former compli- could be solved by clarification of tubes by a brief centrifugation. The complication was corrected by the re-l assays with variously diluted samples r by washing the antigen–antibody exes in the 33% saturation of $_2SO_4$. The hemolytic or lipemic sera did fect the results of the IEA method.

other experiments, we tried a second dy method (goat anti-rabbit IgG anti-bodies) to precipitate soluble immune complexes to compare the quantitative recovery of immune complexes. The second antibody method gave comparable results. However, the method required a longer incubation period and was more expensive.

In order to investigate the correlation of PAP values between the IEA method and the double-antibody RIA, we assayed 40 normal male sera (age 48–66) and 25 clinical serum samples of female patients (Departments of Obstetrics and Gynecology and Oncology; age 35–55) for serum PAP levels by both methods. The normal serum PAP values were 10.5 ± 6.5 ng/ml (range 2.5 to 68 ng/ml) for males and 3.5 ± 2.8 ng/ml (range 2.5 to 9.5 ng/ml) for females. These values are comparable to the normal value (16.0 ± 8.0 ng/ml; range 2.5 to 48 ng/ml) we have reported for 162 normal adult males (8). The normal serum PAP values were 10.0 ± 4.4 ng/ml (range 0.00 to 37 ng/ml) for males and 10.5 ± 4.5 ng/ml (range 0.00 to 18 ng/ml) for females by the IEA method. Therefore, the normal values of serum PAP for males and females by the IEA method are comparable to those obtained by the double-antibody RIA. Fifty serum specimens from patients diagnosed as having prostatic carcinoma in various clinical stages were assayed for PAP by the double-antibody RIA and by the IEA method. With few exceptions (7 out of 50), assay values correlated closely (Fig. 5). It is not clear at this moment whether the discrep-

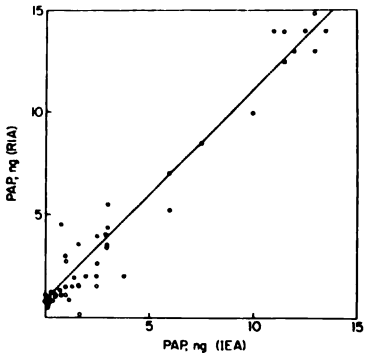

FIG. 5. Unknowns in serum specimens of prostatic cancer patients. Comparison between the IEA and the double-antibody RIA. The equation of the regression line is $y = 1.02x + 0.82$ (y = the double-antibody RIA of Choe *et al.* (8), x = the IEA method, $r = 0.9993$, $n = 50$, $p < 0.0025$).

ancies in these seven cases reflect the occurrence of variant of PAP synthesized by tumor cells or suggest differential inactivation of various PAP isozyme subpopulations by each antiserum. Theoretically, RIA can measure proteins which are immunologically cross-reactive with PAP but devoid of any enzyme activity. Traditionally, enzyme levels are determined as activity rather than as protein. Activity measurement is proportional to the enzyme protein only when the kinetic parameters of enzyme molecules are identical. It is not established yet that kinetic parameters of acid phosphatases from various sources, including neoplastic tissue, are identical. Actually their K_m and V_{max} could be widely different. Therefore, it is possible that the two immunoassay values may not correspond to each other in all cases.

IEA offers several advantages over the RIA method in terms of safety, simplicity, and cost. Problems inherent in the RIA method are the short shelf life of radioisotope-labeled antigen, relatively lengthy assay steps, spillage of radioactive reagents, and cost of assay. On the other hand, the IEA method has a few unique problems which will limit its general application. When a large number of samples were assayed manually for enzymatic activity, experimental errors are magnified by improper temperature equilibration, mixing of substrate, and inconsistency of incubation period. Automation may increase the precision of the assay. Furthermore, there is a possibility that some of the antibodies might block the catalytic site of the enzyme molecule and so could not be used in the technique described.

Summary. Human sera or plasma containing acid phosphatases were mixed with rabbit antiserum specific for prostatic acid phosphatase (PAP) and the resulting primary immune complexes were precipitated by ammonium sulfate. The amount of specifically p tated PAP was estimated directly by zyme activity. Using this assay, as littl ng/ml of the PAP could be determine cellent correlation of this assay metho other immunoassays of PAP was d strated.

This work was supported in part by PHS R Grant CA 18748 and CA 16426 from the N Cancer Institute.

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The Influence of 1-Amino-1-Cyclopentane Carboxylic Acid (Cycloleucine) on Food Efficiency in Rats (40691)[1]

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Cycloleucine, chemically known as 1-aminocyclopentane-1-carboxylic acid (ACPC) is not a naturally occurring amino acid (see Figure 1). Metabolic studies of ACPC in mice and rats have shown that it is not metabolized nor excreted rapidly; therefore, blood and peripheral tissue levels are maintained for several days (1, 2). Ross et al (3) reported a decreased growth rate in animals fed a diet containing ACPC and suggested it may function as an amino acid antagonist. Clark et al (4) proposed that its effect on growth rate and amino acid pools were secondary to a depressed food intake.

The results obtained in the present study suggest yet another mechanism of action of ACPC on reducing food efficiency in rats fed a purified high starch diet.

Materials and methods. Male Wistar rats (160–170 g) were given a standard laboratory Purina Chow or high carbohydrate diet[2] in powdered form *ad libitum* to facilitate estimation of daily food consumption. They were housed individually in open wire bottom cages. Water was available at all times. In experiment I, 5 days after commencement of the high carbohydrate diet, groups of animals received either the basal ration or ACPC incorporated into the diet at the following levels: 0.0125, 0.025, 0.05, or 0.1%. In experiment II, ACPC and its chemically related amino acids (L-(−)-proline, L-4-hydroxyproline, glycine, L-leucine, or L-isoleucine) were admixed separately to the Purina Chow diet at 0.5 mg/g diet (0.05%) or 1 mg/g diet (0.1%), respectively. Animals were given *ad libitum* for a period of 3 days.

Body weight and food consumption were monitored throughout experimental period. Blood was obtained by cardiac puncture and portions of the pancreas, small intestine, and liver were collected and chilled rapidly from anesthetized rats at the end of the studies.

Serum sugar and insulin determinations. Serum levels of reducing sugars were measured with a Technicon Auto Analyzer (Technicon Method N-2b) whereas insulin levels were estimated by radioimmunoassay (New England Nuclear).

Determination of amylase activity. Pancreas and liver sections were weighed and homogenized in cold 0.9% sodium chloride. Whole homogenates were used for α-amylase estimation. A microadaptation of the starch–iodine method of Street (5) was used for amylase activity determination. A 1% soluble starch was dissolved in 20 mM phosphate buffer at pH 6.9 containing 6.9 mM NaCl by heating. Test tubes containing the diluted enzyme solution and buffer to a final volume of 0.4 ml were placed in a water bath (37°) for 3–5 min. A sufficient amount of the starch solution was at the same time placed in another test tube in the water bath.

Subsequently 0.1 ml of the starch solution was transferred to the tubes containing the diluted enzyme. The solutions were mixed and at the same time a stopwatch was started. After exactly 3 min of incubation, the absorbance of the starch–iodine complex obtained on addition of 0.1 ml digest to 5.0 ml of 0.02% iodine–0.2% KI solution was measured in a Bausch & Lomb Spectronic 20 at 680 nm. The unit of amylase activity is expressed as the amount of enzyme sufficient to hydrolyze 1 mg of starch in 3 min at 37°.

Assay of disaccharidase activity. A 15-cm segment of small intestine (the first portion of 5-cm segment was discarded) was separated and flushed with 3 to 5 ml of cold distilled water.

This segment was cut open and laid on a

[1] Part of this work was presented at the First Biochemistry Section Meeting of the New Jersey Academy of Science at Camden, N.J. in April 1976.

[2] High-carbohydrate diet, obtained from Nutritional Biochemical Co., Cleveland, Ohio with the following composition: "vitamin-free" casein 18%; starch 68%; vegetable oil 8%; brewer yeast U.S.P. 2%; salt mixture U.S.P. No. 2, 4%; plus ICN vitamin diet fortification mixture.

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cold sheet of glass placed on crushed ice. The mucosa was scraped off with a microscopic glass slide and homogenized with 4.0 ml cold distilled water. The test tubes were chilled with crushed ice before and during homogenization. Nuclei and larger cell debris were removed by centrifugation at 500 *g* for 20 min at 4°.

The supernatant contained the disaccharidases. Maltase, sucrase, and lactase activities were assayed by the Tris–glucose oxidase method as described by Dahlqvist (6). One unit of disaccharidase activity is expressed as micromoles of substrate hydrolyzed per minute.

Pancreatic lipase activity. Lipase was prepared by extracting rat pancreas ethanol–acetone powder with water. The supernatant solution obtained upon centrifugation was used to assay for lipase activity according to Marchis-Mouren *et al.* (7) by the use of a pH-stat (Radiometer titrigraph). Protein was determined according to Lowry *et al.* (8), using Folin–Ciocalteau reagent. The specific activity of a lipase solution is defined as the number of lipase units/mg protein.

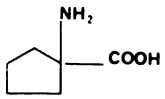

FIG. 1. The chemical structure of cycloleucine—ACPC.

Results and discussion. Control rats in experiment I gained an average of 28 g during the experimental period. Animals receiving ACPC in their diet showed a dose-related loss in body weight gain (Table I) associated with reduced food efficiency (Fig. 2). Food consumption, however, was not significantly reduced except at the highest (0.1%) concentration of ACPC in the diet. Levels of serum sugar, insulin, and amylase activity were not altered by ACPC treatment (Table II).

Pancreatic α-amylase activity was markedly reduced at all levels of ACPC treatment but was statistically significant at the two highest concentrations only (Table III). Conversely, hepatic α-amylase activity increased in a dose-related manner being significant at the three highest concentrations of ACPC in the diet (Table III). The overall hepatic α-amylase activity was found to be relatively small compared with that of the pancreas.

Table IV shows the intestinal disaccharidase activities in rats fed a high carbohydrate diet containing 0.1% ACPC. The activities of all three hydrolases (maltase, sucrase, and

TABLE I. EFFECT OF ACPC ON WEIGHT GAIN AND FOOD CONSUMPTION IN RATS (3 DAYS ON PURIFIED DIET)

% of ACPC in diet	Number of rats	Weight gain—3 days		Food consumption—3 days	
		g (mean ± SEM)	Change as % of control	g (mean ± SEM)	Change as % of control
0	5	28.0 ± 1.7	—	63.6 ± 2.9	—
0.0125	5	22.4 ± 2.4	↓20	56.2 ± 3.7	↓12
0.025	6	20.7 ± 2.1	↓26*	60.0 ± 2.8	↓ 6
0.05	6	19.7 ± 4.3	↓30*	60.2 ± 5.3	↓ 5
0.1	5	10.2 ± 1.7	↓64**	48.3 ± 2.5	↓24**

* Statistically significant from control, $P \simeq 0.01$.
** Statistically significant from control, $P < 0.001$.

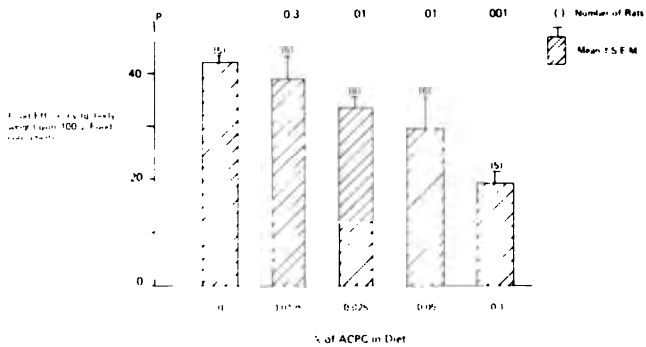

FIG. 2. The effect of ACPC on food efficiency in rats.

TABLE II. EFFECT OF ACPC ON SERUM SUGARS, INSULIN, AND α-AMYLASE

% of ACPC in diet	Number of rats	Serum sugars mg% (mean ± SEM)	Serum insulin μ units/ml (mean ± SEM)	Serum α-amylase activity units/ml (mean ± SEM)
0	5	167 ± 5	50 ± 2	20.42 ± 0.64
0.0125	5	172 ± 7	50 ± 17	20.20 ± 1.50
0.025	6	163 ± 3	52 ± 7	19.79 ± 0.89
0.05	6	168 ± 4	55 ± 8	19.44 ± 0.62
0.1	5	165 ± 2	45 ± 5	18.89 ± 1.13

lactase) were not altered by ACPC treatments. However, pancreatic lipase was reduced to about a third of that observed in the absence of ACPC but was not statistically significant from the control.

Results obtained in experiment II are presented in Table V. A decrease in food efficiency was not observed when other amino acids were incorporated into the diet. These results are in contrast to those obtained when ACPC was incorporated into the diet.

TABLE III. EFFECT OF ACPC ON PANCREATIC AND HEPATIC α-AMYLASE ACTIVITIES

		Pancreatic α-amylase activity		Hepatic α-amylase activity	
% of ACPC in diet	Number of rats	units/100 mg tissue (mean ± SEM)	Change as % of control	units/100 mg tissue (mean ± SEM)	Change as % of control
0	5	588 ± 76	—	0.54 ± 0.05	—
0.0125	5	398 ± 90	↓32	0.69 ± 0.14	↑ 28
0.025	5	518 ± 71	↓12	1.47 ± 0.21	↑172**
0.05	6	336 ± 27	↓43*	1.59 ± 0.17	↑194**
0.1	5	241 ± 21	↓59**	3.64 ± 0.74	↑574**

* Statistically significant from control, $P \simeq 0.01$.
** Statistically significant from control, $P < 0.001$.

TABLE IV. EFFECT OF ACPC ON RAT INTESTINAL BORDER DISACCHARIDASES AND PANCREATIC LIPASE

	Disaccharidases[a]						Lipase[b]	
	Maltase		Sucrase		Lactase			
Treatment	units/g protein (mean ± SEM)	Change as % of control	units/g protein (mean ± SEM)	Change as % of control	units/g protein (mean ± SEM)	Change as % of control	units/mg protein (mean ± SEM)	Change as % of control
Control	275 ± 23	—	61 ± 5	—	7.8 ± 1.5	—	3.2 ± 0.62	—
ACPC 0.1%	227 ± 16	↓17	73 ± 5	↑20	7.1 ± 1.1	↓9	2.2 ± 0.50	↓31

[a] Each treatment consists of six rats.
[b] Each treatment consists of five rats.

TABLE V. EFFECT OF DIFFERENT AMINO ACIDS ON BODY WEIGHT GAIN, FOOD CONSUMPTION, AND FOOD EFFICIENCY (3 DAYS ON PURINA CHOW)

		Body weight gain—3 days		Food consumption—3 days		Food efficiency	
Amino acids	% in diet[a]	Grams (mean ± SEM)	Change as % of control	Grams (mean ± SEM)	Change as % of control	Grams body weight gain/100 g food (mean ± SEM)	Change as % of control
None	—	28 ± 2	—	76 ± 3	—	37 ± 3	—
Cycloleucine (ACPC)	0.05	13 ± 3	↓54***	62 ± 5	↓18*	21 ± 5	↓43**
L-(−)-Proline	0.10	26 ± 2	↓ 7	71 ± 3	↓ 7	36 ± 2	↓ 3
L-4-Hydroxyproline	0.10	27 ± 3	↓ 4	76 ± 2	0	36 ± 4	↓ 3
Glycine	0.10	27 ± 1	↓ 4	79 ± 3	↑ 4	40 ± 7	↓ 8
L-Leucine	0.10	25 ± 2	↓11	73 ± 2	↓ 4	35 ± 2	↓ 5
L-Isoleucine	0.10	29 ± 2	↑ 4	75 ± 2	↓ 1	35 ± 3	↓ 5

[a] Each treatment consists of six rats.
* Statistically significant from control, $P \simeq 0.05$.
** Statistically significant from control, $P \simeq 0.01$.
*** Statistically significant from control, $P < 0.001$.

Clark and his co-workers (4) showed that the decreased growth rate of ACPC treated rats was secondary to the effect on reduced food intake when animals were on a high glucose (65.8%) diet. In other words, reduction of body weight gain was seen only when the food consumption was affected. In our experiment, decrease in body weight gain of ACPC-treated rats (0.0125–0.05% in the diet) was not related to reduced food intake. However, 0.1% ACPC caused a marked decrease in body weight and a weak to moderate effect on food consumption.

The activities of other digestive enzymes such as intestinal maltase, sucrase, lactase, and pancreatic lipase were not significantly affected by ACPC treatment. However, production of pancreatic α-amylase is essential for converting starch into maltose which is broken down into glucose, the predominant form of carbohydrate prior to being absorbed in the gastrointestinal tract. Retardation or inhibition of pancreatic α-amylase by ACPC would reduce food efficiency by limiting starch digestion thereby reducing available glucose for absorption causing caloric imbalance resulting in the loss of body weight. In the study reported by Clark *et al.* (4), glucose was present in the diet as the carbohydrate source, and α-amylase would have no influence on the glucose absorption. Therefore, the reduction of body weight gain in rat maintained on glucose-rich diet was probably due to the lowering of food intake. In the current study the loss of body weight and lowering of food efficiency may be attributed, in part, to the reduction of pancreatic α-amylase caused by ACPC.

It was also interesting to note that the serum insulin level was not affected by ACPC treatment, an indication that at least the β-cells of the pancreas were not effected in rats after 3 days of ACPC treatment. The increase of α-amylase activity in the livers of ACPC-treated rats was an unexpected response. At this time, we do not have any explanation for this observation.

Chemically related amino acids, proline, hydroxyproline, glycine, leucine, and isoleucine did not show any influence on food efficiency in rats. The lowering of pancreatic α-amylase activity in ACPC-treated rat is considered the specific cause of the decrease of food efficiency in this study.

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ılcium, Amylase, and Flow Rate of Rat Parotid Saliva with Diverse Frequencies of Parasympathetic Nerve Stimulation (40692)[1]

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lcium concentrations of parasympa-
ally evoked saliva are generally re-
d to be lower than those of sympatheti-
evoked saliva (1). However, recent evi-
e shows that when electrical stimuli of
ımaximal intensity are used to stimulate
arasympathetic innervation to rat pa-
, the evoked saliva has a high concentra-
of calcium, as well as a high rate of flow
ince sympathetically evoked saliva (es-
lly with isoproterenol) from rat parotid
has a similarly high concentration of
ım but a much lower flow rate than
ympathetically evoked saliva (3), it be-
important to determine if calcium con-
ation of saliva is in fact independent of
rate, especially since some reports indi-
a dependence of calcium concentration
ow rate, while other reports show the
site relation (4, 5). It is believed that
controversial differences have arisen
ıse comparisons were made without re-
to kind of stimulation employed (6).
a particular kind of autonomic stimu-
ı, concentration of calcium may be de-
ent on flow rate. Therefore, the present
was undertaken to determine the rela-
hip between calcium concentration and
rate with a single kind of autonomic
ılation. The postganglionic parasympa-
innervation to rat parotid can be stim-
d electrically, and intensity of stimula-
an be altered by varying the frequency
ectrical impulses to the nerve. By this
dure, flow rate also is readily varied and
ases with increases in frequency (6).
ablishment of the relationship between
ım and flow rate is important for a
er of reasons. First, if, like Na^+, it is
l to be dependent on flow rate, or like
ndependent of flow rate, inferences re-
ng transport of this ion can be made (5, 7, 13). Second, it has been suggested that secretion of calcium is closely bound to secretion of the enzyme amylase (8). However, while this appears to be true under some conditions of stimulation (1, 9), a second pathway for calcium secretion that is not linked to amylase secretion may occur under conditions of stimulation of the parasympathetic nerve (2). Thus, determination of the influence of flow rate on amylase as well as calcium concentration of saliva may provide further evidence for existence of a second pathway for calcium secretion that is not linked to secretion of amylase.

Materials and methods. Long–Evans adult female rats, used in these experiments, were maintained on standard lab chow and water *ad libitum* until 18 hr before experimentation, when food but not water was withdrawn. Rats were anesthetized with 1% Nembutal and the parotid duct and auriculotemporal nerve were then exposed. The duct was cut at its anterior end, and micropipets, applied to the cut surface, were used to collect measured volumes of fluid during a timed interval. This provided a measurement of flow rate. Since gland weight could not be determined at each successive point of stimulation, flow rate was more conveniently expressed as μl/min.

Flow of saliva was elicited by continuous stimulation of the parasympathetic nerve (auriculotemporal) to the parotid for at least 80 min. A Grass stimulator was used to deliver square wave pulses (4 V, 4-msec duration) at frequencies varying from 5 to 20 Hz (7).

Since it had previously been shown that 20 Hz represented an intensity that was supramaximal (7), frequencies above this were not investigated. The previous work (7) also showed that with 60 min of continuous stimulation at the single supramaximal frequency of 20 Hz, flow rate remained consistently high for about 30 min but gradually decreased thereafter. It was, therefore, necessary to eliminate changes that appeared with in-

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creasing time so that the influence of frequency of stimulation on flow rate could be more precisely assessed. The present kinds of experiments were thus performed so that this could be accomplished. Thus, frequency of stimulation was changed sequentially in the same rat (total rats used, 12) from low (5 Hz) to high (20 Hz) and then from 20 to 5 Hz. While these changes in frequency were in each case done in the same animal, the order of change of frequency was varied from animal to animal. Duration of stimulation in these experiments at each frequency was usually about 10 min; before a sample of saliva was collected at a new frequency, an interval of at least 5 min was allowed to elapse. This was more than ample (3 min in fact was adequate) to establish a steady state (accounting for gland and duct lag in clearance of saliva) and the new values obtained were, therefore, representative of the effect of the new frequency.

Twelve animals were examined and for three of these, the flow rate, calcium concentration and amylase activity of saliva elicited at each frequency of stimulation were examined over a period of 80 min of continuous nerve stimulation. In one additional rat, stimulation was maintained for 120 min and since gland levels of amylase change within this time, and the saliva concentrations reflect these changes, amylase of saliva was not determined in this experimental animal (1D). With eight additional rats, the parameters mentioned were examined at particular frequencies and during a specific period (initial 40 min after initiation of nerve stimulation). These provided mean values for each parameter with each frequency of stimulation.

Amylase activity was determined by the method of Myers *et al.* (10) and expressed as milligrams of reducing substance formed per microliter of fresh saliva in 15 min at 37°C.

Calcium concentration of saliva or serum samples was determined using a Fiske titrator. Comparison with values obtained using atomic absorption method for calcium determination showed that the values from the two methods agreed well.

Results. Table I includes data from eight rats and shows the mean values for flow rate, calcium concentration and amylase activity of the saliva during stimulation of the auriculotemporal nerve at frequencies of 5, 10, 16, and 20 Hz; furthermore, these values are those obtained during the early period following initiation of continuous nerve stimulation of 80-min duration. Duration of stimulation in these experiments at each frequency was usually about 10 min; the details for stimulation and collection of saliva have already been described. The data show that flow rate and calcium concentration rise with sequential increases in frequency of stimulation, with mean increments in flow rate of approximately 6 μl/min (eight rats) and mean increments in calcium concentration of 3–4 meq/liter evident when frequency was increased from 5 to 10 Hz, and then from 10 to 16 Hz. These represented increases of at least 50% in flow rate and calcium when frequency was changed from low to high. In fact in a specific instance (when frequency of stimulation was increased from 5 to 10 Hz), there was a 1.5-fold increase in flow rate. Conversely, when frequency was decreased from high to low, flow rate and calcium concentration were decreased.

Amylase concentration on the other hand was independent of flow rate and the values of approximately 16 mg/μl of saliva represent the activity of this enzyme at all flow rates

TABLE I. EFFECTS OF VARYING FREQUENCY OF STIMULATION OF AURICULOTEMPORAL NERVE ON FLOW RATE AND CALCIUM AND AMYLASE CONCENTRATIONS OF RAT PAROTID SALIVA[a]

Frequency (Hz)	Flow rate (μl/min)	Calcium conc. (meq/liter)	Amylase activity (mg/μl of saliva)
5	4.5 ± 0.38	5.7 ± 0.31	15.6 ± 1.81
10	11.2 ± 1.70	9.1 ± 0.25	16.4 ± 1.56
16	17.1 ± 2.21	13.8 ± 0.26	17.2 ± 1.61
20	16.8 ± 2.10	12.7 ± 0.23	16.0 ± 0.98

[a] Values are means ± SE; total number of rats was eight. Flow rate is expressed as volume/min (μl/min), and amylase concentration as milligrams of reducing substance formed in 15 min at 37°C. Frequency of stimulation is expressed as Hz. Values for flow rate and calcium concentration (but not amylase) at 5 and 10 Hz are statistically different from those at 16 and 20 Hz ($P < 0.001$); those at 5 differ from those at 10 Hz, and those at 10 differ from those at 16 Hz ($P < 0.001$), but again amylase values are the same at all frequencies. Values for calcium concentration and flow rate at 16 and 20 Hz are virtually identical. Mean calcium concentrations of serum from unstimulated rats were 4.9 ± 0.4 meq/liter (eight rats), and those of serum obtained after 80 min of nerve stimulation were 4.6 ± 0.5 meq/liter (eight rats).

ncies of stimulation examined (Ta-
'ig. 1).
ı in Fig. 1 confirm and extend this
ı. Data from 4 of the 12 rats used
periments are presented separately
ıvidual values at each flow rate for
r animal can be examined during
30 min of continuous nerve stimu-
ne of these, stimulation was con-
as long as 120 min but no amylase
e determined on saliva from this
D) since the concurrent gland de-
this enzyme over this extended
ime would modify amylase levels
ıd yet not be related to changes in
Other additional points are also
by data in Fig. 1.
r, e.g., that the changes in calcium
ıte are very sensitive to changes in
of stimulation not only when fre-
sequentially increased but also
sequentially decreased. Further-
e changes are induced rapidly in
ınimal and this alteration can be
for a long period of time. The
of altering frequency sequentially
: animal over a long period of time
y been alluded to in Materials and
Thus, when effects due to time
(within 30 min after initiation of
ı), the effects of frequency on flow
lcium concentration become ob-
these time-induced changes. In
ent experiments, even if absolute
ılcium or flow have decreased as a
ce of time to a new lower level, and
for example, a frequency of 16 Hz
:auses as high a calcium concentra-
ow rate as initially induced by this
from this new, lowered-base line,
in frequency still brings about a
calcium or flow and it is usually
magnitude to the original changes

ı levels of serum obtained from the
d experimental animals were 4.6
/liter and 4.9 ± 0.3 meq/liter re-
(after 8 rats) and did not differ
other ($P < .001$). For control val-
ı was obtained from unstimulated
he experimentals, serum was ob-
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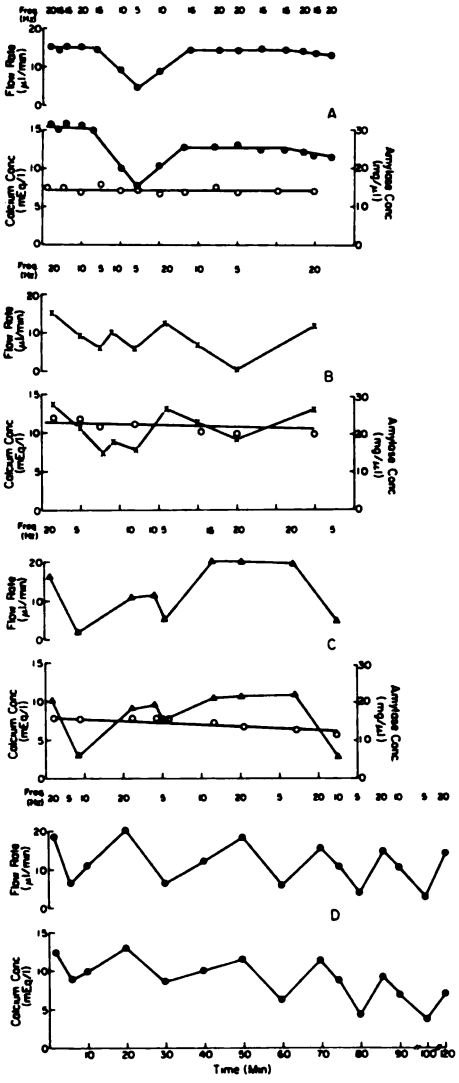

FIG. 1. The data in Fig. 1 show the relationship between frequency of stimulation, flow rate, calcium concentration, and amylase concentration. Each of the four graphs (A–D) represent individual values from four separate rats. Data were collected over a period of at least 80 min (120 min for 1 D) when continuous electrical stimulation of the auriculotemporal nerve was employed. Flow is expressed only as vol/min since the same gland was being stimulated in each case. Black signs are used to represent calcium concentration and flow rate; open circles are used to show amylase activity (expressed as mg reducing substance/μl of saliva). Frequency variation is presented horizontally by appropriate numbers at the top of each of the four experiments, and time (in min) at the bottom. Amylase activity was not determined for animal 1 D.

The calcium concentration of saliva was not statistically different from that of serum at a frequency of 5 Hz, but double that of serum at 10 Hz, and three times that of serum at 16 or 20 Hz.

Discussion. The frequency of stimulation of the parasympathetic nerve to parotid gland of rat is an important factor in regulating flow rate and calcium concentration of the nerve-evoked saliva but has no influence in regulation of amylase concentration of the saliva. Both calcium concentration and flow rate increase with increases in frequency of stimulation, until a plateau is reached at 16 Hz. From these observations it may be concluded that flow rate has a regulating influence on calcium concentration of parasympathetically evoked saliva but the occurrence of a plateau suggests that the ability of gland cells to absorb or secrete calcium has a fixed limit. Thus, salivary calcium levels are in part also regulated by this fixed capability of the cells as well as by flow of the saliva. However, these conclusions must be considered valid only when a particular kind of autonomic stimulation is employed. When flow rates and calcium concentrations of saliva evoked by two different kinds of autonomic stimulation are compared, flow rate has no role in accounting for differences in calcium concentration. It has been shown, for example, that isoproterenol (3) or stimulation of the sympathetic nerve (6) can elicit saliva of very low flow rates (4 μl/min). Yet the calcium concentrations of such salivas are usually as high (11–13 meq/liter) (3) or nearly as high (11 meq/liter) (6) as that observed with high rates of flow induced by high frequency of stimulation of the parasympathetic nerve (11–13 meq/liter), and are not at all comparable to the low calcium concentrations (5 meq/liter) observed at low, parasympathetically induced, flow rates (4 μl/min at 5 Hz).

From the present data some inferences can be made concerning the mechanism by which salivary concentration of calcium is regulated and what bearing flow rate has on this. For example, at the highest flow rates, the final saliva does exhibit a calcium concentration that is three times higher than that of serum (11). One possibility to account for this would assume that at the acinar cell level a precursor fluid rich in calcium is produced, and that modification in calcium concentration does not occur as this fluid rapidly passes thr the ducts (12). The contact time of the cium-rich precursor fluid with duct ce too brief at the highest flow rates to p any reabsorption of calcium; thus conce tions in the final saliva are high (12). versely, at lower flow rates, where co time between duct cells and calcium-rich cursor fluid is prolonged, more calciu reabsorbed, and the calcium concentrati the final saliva is much lower (11, 12). V flow rate thus has an important effe regulating calcium concentration of saliv a particular kind of autonomic stimulati does not have exclusive control over it.

It must be made clear that the abov guments assume that a calcium-rich prec fluid is formed at the acinar level and t flow-related reabsorption of calcium o at the ductal level. This assumption s most logical on the basis of present dat several reasons: (i) calcium concentrati the final saliva is three times that of se (ii) since calcium concentration increases increased flow rate, rather than decre with increasing flow, secretion of cal from duct cells into a final saliva is not li furthermore, recent unpublished data on perfusion show that calcium is reabso (not secreted) by duct cells of submaxil However, definitive resolution depend examination of calcium concentration o precursor fluid. Until that is accompli other possibilities must be considered. example, the calcium concentration of precursor fluid may be similar to tha serum, but increases as the precursor passes through the duct system, where cium is secreted by duct cells and calciu the final saliva thereby is increased. Su mechanism could thus also result in cal levels in the final saliva that are three t higher than that of serum. (iii) Water r sorption by ductal cells could also acc for this threefold increase in calcium of s (when compared with serum levels). Or basis of other evidence, this does not ap likely, however. Thus, it has been show perfused main duct of rat submaxillary g that the duct cells are relatively imperme to water (5). In general, the behavior of cium with flow rate makes the first possib more tenable.

Amylase is independent of changes in

ıd remains the same at all levels of ıtion. Since changes in flow rate pro- :hanges in calcium concentration, it e concluded that at least some fraction total secreted calcium must not be to amylase. This is not in contradiction generally held view that when amylase eted, it is secreted with calcium (14). 'er, calcium may be secreted into saliva .t amylase bound to it under certain ıons of stimulation (11).

mary. Calcium concentration of saliva, electrical stimulation of the parasym- ıc postganglionic fibers to rat parotid is o evoke secretion, increases with in- ; in flow rate, but both calcium concen- and flow rate reach maximal levels at ncies of 16 Hz. The concentration of n at highest flow rate is three times ' serum. Amylase activity of the saliva ıs consistently low at all flow rates. ore, these data show that while calcium nylase are secreted together, some cal- nay be secreted without amylase. By ıce it is also possible to suggest that the ıtrating mechanism for calcium is at inar level, and that as the precursor ich in calcium, flows through the duct , very little absorption of calcium by cells occurs at high flow rates, but as t time is increased with decreasing flow ıore calcium is reabsorbed, resulting in saliva low in calcium. It is not likely ıe increased calcium concentration is ult of reabsorption of water by ductal nce in perfused main submaxillary duct t, the duct cells are relatively imperme-) water. The present data suggest this etation, but micropuncture analysis of n concentration of the precursor fluid is required to establish this as a fact. Flow rate differences found with different kinds of autonomic stimulation cannot be used to assess relationships between calcium concentration and flow rate of saliva, and only when the flow rate and calcium concentration are compared with a particular kind of autonomic stimulation are such comparisons meaningful.

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The Methanol Extraction Residue of *Bacillus Calmette–Guerin* Protects against 7,12-Dimethylbenz(α)anthracene-Induced Rat Mammary Carcinoma (40693)[1]

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The carcinogen, 7,12-dimethylbenz(α)-anthracene (DMBA), induces mammary adenocarcinoma in Sprague–Dawley female rats when administered by stomach tube. Tumor incidence depended on the quantity and type of dietary fat (1–4). Rats maintained on diets containing high levels of polyunsaturated fat were more susceptible to tumor development than rats maintained on diets containing similar levels of saturated fat. Rats maintained on low-fat diets were least susceptible as measured by tumor incidence, latent period, number of tumors per tumor-bearing rat, and rate of tumor growth.

Other studies have indicated that tumor incidence can be reduced by treating rats either before or after exposure to DMBA. For example, Takeda *et al.* (5) observed an increase in the latent period and a reduction in tumor incidence if diets fed after DMBA were supplemented with arginine. Similar results were observed when antioxidants were added to diets (6–8).

More recently, Weislow *et al.* (9) reported that rats were protected against DMBA-induced mammary carcinoma by immunization with a mouse xenotropic type C virus, before exposure to DMBA. The mouse virus used in their studies acted as a nonspecific immunostimulant. Spleen lymphocytes were much more responsive to PHA when taken from immunized rats compared to nonimmunized controls. Furthermore, DMBA-induced lymphocyte anergy was prevented by immunization.

The methanol extraction residue (MER) of *Bacillus* Calmette–Guerin is a nonspecific immunostimulant which has been used in both prophylactic (10–12) and therapeutic (13, 14) animal tumor models. Since MER potentiates the immune response to microbial pathogens, antigens, and tumor cells, experiments were designed to determine if MER provided prophylactic or therapeutic benefit in Sprague-Dawley female rats exposed to DMBA. Rats maintained on different fat diets were used to determine if protection was influenced by dietary fat.

Materials and methods. Animals. Sprague-Dawley female, albino, outbred rats were used for these studies. Weanlings (21 days old) were obtained from the Charles Rivers Colony (Willmington, Mass.) or from Charles Rivers Breeders maintained at the University of Oklahoma Health Sciences Center (Oklahoma City, Okla.). Rats were maintained (*ad libitum*) on one of three diets (ICN Pharmaceutical, Inc., Life Sciences Group, Cleveland, Ohio) outlined in Table I. Based on calories per gram of diet and the average amount of food consumed per day, it was estimated that the daily caloric intake was similar for all groups. Growth curves indicated no differences between rats on the high- and low-fat diets.

Exposure to DMBA. Rats which were exposed to the carcinogen were given 10 mg of DMBA (Sigma Chemical Co., St. Louis, Mo.) in 1 ml of stripped corn oil via stomach tube at 50 days of age. Animals were weighed and examined for tumors on a weekly basis. In experiments where rats were immunized with MER (National Cancer Institute, Silver Springs, Md.) before exposure to DMBA, rats were killed when the experiment was terminated (32 to 34 weeks after DMBA). Tumors were removed and certified histologically as

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ABLE I. CONTENT OF VARIOUS DIETS

	Polyunsaturated fat diet (g)	Saturated fat diet (g)	Low-fat diet (g)
	23	23	23
	20[a]	20[b]	2[c]
	46	46	64
re[d]	4	4	4
(nonnutrient	6	6	6
ixture[e]	1	1	1

ed corn oil.
ed, hydrogenated coconut oil, 18% + linoleic
ic acid.
ixture (15).
in mixture (vitamin fortification mixture of Sciences Co., Cleveland, Ohio 44128).

y adenocarcinoma. Mammary adeoma did not appear in rats which t exposed to DMBA. Experiments determine the therapeutic value of still in progress. Observations have de for 28 weeks after exposure to

ization with MER. MER, suspended e (0.5 mg/0.2 ml) was injected intrally (0.5 mg/injection, all immunized given two injections). Control anire given 0.2 ml of vehicle, via the ute, which contained the following liter of water: 9 mg sodium chloride, dium carboxymethylcellulose, 0.004 olysorbate, and 0.009 ml of benzyl Rats given MER before exposure to were immunized at 28 and 35 days n therapy studies, rats were placed olyunsaturated fat diet at time of and maintained on these diets for or 4 weeks after DMBA. At each of ies, rats were assigned to one of four One group was maintained on the turated fat diet and not treated. A group was maintained on the polyted fat diet and treated with MER. s given either at 3 and 5 weeks after or at 4 and 6 weeks after DMBA. A up was placed on the low-fat diet. A roup was placed on the low-fat diet ed with MER either at 3 and 5 weeks MBA or at 4 and 6 weeks after

ical analysis. The χ^2 statistics were compare final tumor incidence between immunized and nonimmunized rats in each dietary group, and 28-week incidence between therapy groups. A median test was performed to compare size of tumor in tumor-bearing rats, between immunized and nonimmunized rats.

Results. Tumor incidence in rats immunized with MER or treated with vehicle before exposure to DMBA is shown in Fig. 1. Figure 1A shows the protective effect of MER in rats maintained on the polyunsaturated fat diet. Rats treated with MER had a longer latent period and a significant reduction in tumor incidence ($P \leq 0.01$) compared to vehicle-treated controls. Figure 1B illustrates similar data for rats maintained on the saturated fat diet. Immunized rats had a significantly lower tumor incidence ($P \leq 0.05$) compared to nonimmunized rats. Data for rats maintained on the low-fat diet is shown in Fig. 1C. MER-treated rats had a longer latent period and a significantly lower tumor incidence ($P \leq 0.01$) compared to the nonimmunized group.

Since MER-treated rats had longer latent periods compared to nonimmunized controls, it appeared that MER provided benefit to all rats, although some rats were protected only temporarily. Further evidence of the prophylactic benefit observed with MER was obtained by evaluating tumor mass in rats with tumor. The median tumor mass of all rats, independent of diet and treatment was 40 g. The proportion of rats with small tumor masses (less than 40 g) was significantly higher in MER-treated rats compared to vehicle-treated controls.

Results from therapy studies are shown in Fig. 2. Since results were similar when therapy was initiated at 3 or at 4 weeks, these data were combined. Results indicated that MER provided no protection when given to rats on the polyunsaturated fat diet. Rats placed on the low-fat diet had a significantly lower tumor incidence ($P \leq 0.01$) compared to untreated rats maintained on the polyunsaturated fat diet. Further therapeutic benefit was observed when rats on the low-fat diet were treated with MER. This group had significantly fewer tumors ($P \leq 0.01$) compared to rats placed on the low-fat diet. Preliminary data indicate that these groups may also be different based on tumor mass and number of tumors.

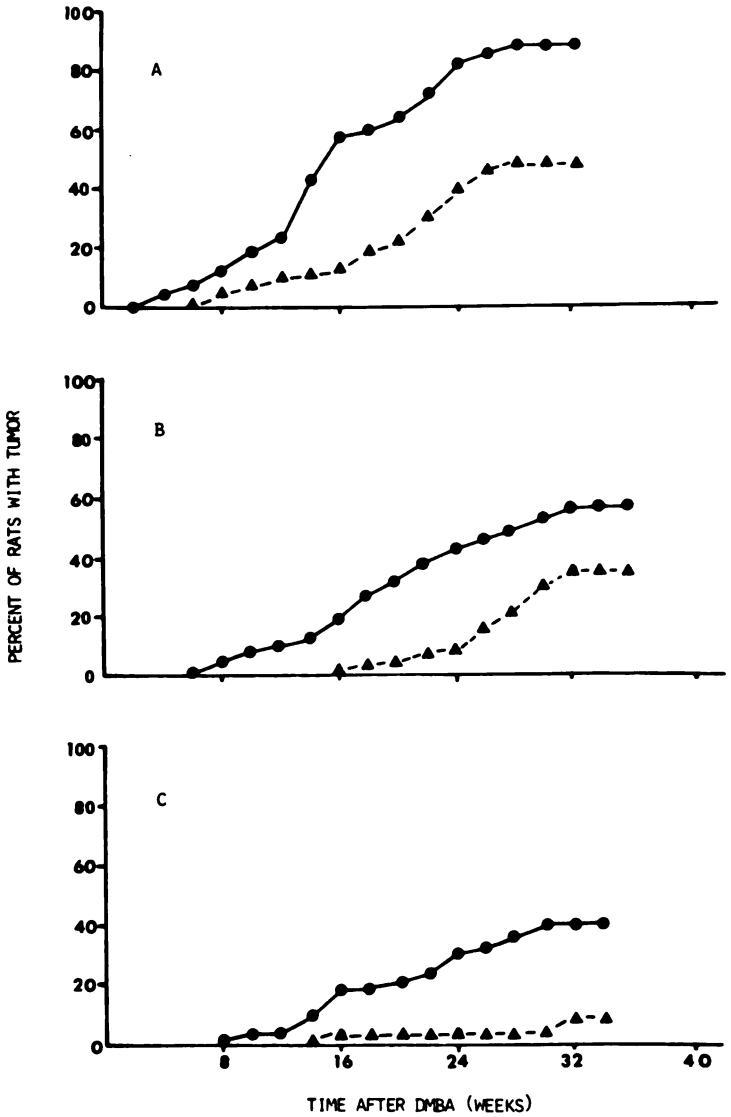
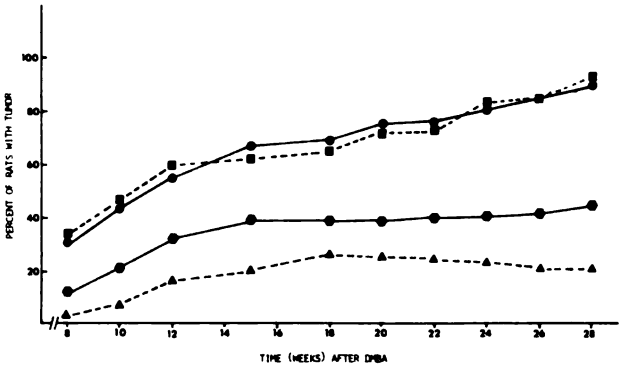


FIG. 1. Rats were maintained on (A) polyunsaturated fat diet, (B) saturated fat diet, or (C) low-fat die immunized with MER or treated with vehicle at 28 and 35 days of age. DMBA was given on Day 50. T incidence is shown as a function of time after DMBA. MER-treated rats (▲) had a significantly low tumor inci compared to rats treated with vehicle (●). Groups consisted of 26 to 30 rats.

Discussion. Results from these studies indicated that MER provided both prophylactic and therapeutic benefit in this tumor–host system. Prophylactic benefit was measured by a reduction in tumor incidence, an increase in the latent period, and a reduction in tumor mass. Further protection may depend on the dose of MER, route of injection, or schedule and frequency of immunizations.

A recent report by Weislow *et al.* (9) i cates that Sprague–Dawley female rats v protected against DMBA-induced mamn adenocarcinoma when immunized wit mouse xenotropic type C virus 7 days be DMBA. Immunization was not effec when given 20 or 30 days before DMB is not possible to determine from their c if this difference in response was due to



2. Rats were maintained on the polyunsaturated fat diet from time of weaning, exposed to DMBA on Day maintained on the polyunsaturated fat diet for either 3 or 4 weeks. At those times, some rats were placed on diet or placed on low-fat diet and treated with MER. Other rats were maintained on the polyunsaturated fat maintained on this diet and treated with MER. Rats placed on low-fat diet (●) had a significant reduction ır incidence compared to rats maintained on the polyunsaturated fat diet (●). Rats placed on low-fat diet and with MER (▲) had a significant reduction in tumor incidence compared to rats placed on low-fat diet (●). rovided no benefit when given to rats on the polyunsaturated fat diet (■). Groups consisted of 26 to 58 rats.

s at the time of immunization or the al between immunization and exposure 1BA.

our study the prophylactic benefit of was observed in all dietary groups, but ɔeutic effect was limited to rats on the ıt diet. While these experiments are still ɔgress, preliminary data indicate that may increase tumor mass when given ɜ on the polyunsaturated diet. Alterna-, rats on the low-fat diet, treated with , has smaller tumor masses compared to n the low-fat diet only.

ɜ observation that tumors could be pre-ɪ, but could not be treated in rats on the nsaturated fat diet, may be due to in-t differences in the experimental de-Rats treated prophylactically were 4 to ɜks old when immunized, while rats therapy were 10 to 11 weeks old when nized. The former group had been on ɔlyunsaturated fat diet for 7 to 14 days ıe of treatment, while the latter group ɜeen on this diet for 50 to 57 days. In on, prophylaxis was given before A, while therapy was given after A when early tumors were probably ıt in all rats. DMBA, like other carcin-, suppresses the immune response (16,

ervations by Carroll and Khor (3) indicate that all rats maintained on the polyunsaturated fat diet had tumors within 3 to 4 weeks after exposure to DMBA. Prolonged feeding of the polyunsaturated fat diet may alter functional properties of lymphoid cells. For example, Stuart *et al.* (18) reported that the functional capacity of the reticuloendothelial system was depressed by simple lipid complexes. Berken and Benacerraf (19) also reported that lipids depressed phagocytosis by the reticuloendothelial system. Since macrophages probably play a major role in regulation of antigen-induced proliferation (20), depression of other lymphoid cells may be mediated through macrophages.

If function of lymphoid cells was inhibited in rats on the polyunsaturated diet, it may have been restored when rats were placed on the low fat. However, the therapeutic benefit associated with the low-fat diet does not necessarily implicate immunological mechanisms. Tumors which developed in rats on the polyunsaturated fat diet may have been selected for their ability to grow in the microenvironment furnished by this diet. Changing to the low-fat diet may select for tumors with different nutritional requirements, analogous to surgical ablation of hormone-producing glands in patients with hormone-dependent breast tumors (21, 22).

However, the observation that MER pro-

duced additional therapeutic benefit when given to rats on the low-fat diet, suggests that these tumors were susceptible to immunological control. Other studies have shown that MER increases the number and distribution of lymphoid cells (23, 24). The uptake of collodial substrates is markedly potentiated in mice pretreated with MER (24). The uptake, degradation, and elimination of foreign protein is also enhanced by MER treatment.

Both intraperitoneal and intravenous injection of MER elicited pronounced elevation in hydrolytic lysozomal enzyme activity of mouse peritoneal macrophages for at least 30 days after treatment (25). Such macrophages also exhibited a greatly amplified ability to phagocytize pathogenic bacteria *in vitro,* notably *staphylococci,* and to inhibit their intracellular replication (26).

If macrophages play a pivotal role in directing lymphoid responses against tumor cells, inhibition of macrophage function via serum constituents (27), which are determined by diets, may jeopardize host immune responses or minimize their benefit.

Summary. Sprague–Dawley female rats were placed on a low-fat diet, a high saturated fat diet, or a high polyunsaturated fat diet at time of weaning (21 days of age). Half of the rats in each dietary group were given an intraperitoneal injection of MER (0.5 mg) on Day 28 and again on Day 35. On Day 50, all rats were given 10 mg of DMBA in 1 ml of stripped corn oil via stomach tube. Tumor incidence was determined weekly by palpation and confirmed by histological analysis upon completion of the experiment. In each dietary group, rats immunized with MER had a lower tumor incidence, a longer latent period, and smaller tumors compared to non-immunized rats. In other experiments, rats were placed on the high polyunsaturated fat diet at time of weaning, exposed to DMBA on Day 50, and maintained on this diet for either 3 or 4 weeks after DMBA. At those times, some rats were placed on the low-fat diet while other rats were maintained on the high polyunsaturated fat diet. Half of the rats from each group were treated with MER. Results indicate that the low-fat diet provided significant therapeutic benefit. Further benefit was observed if these rats had been treated with MER. However, MER was not beneficial if given to rats maintained on the high polyunsaturated fat diet.

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Evidence for Magnesium Secretion during Phosphate Depletion in the Rat (40694)

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Phosphate depletion in the rat is associated with the rapid development of marked magnesuria (1, 2). This phenomenon has been attributed to a decrease in the renal tubular transport of magnesium since the magnesuria occurred in the face of falling filtered load of this ion.

However, it is also possible that magnesium secretion by the renal tubule occurs during phosphate depletion and contributes to the magnesuria. The present study was undertaken to examine this question.

Methods. Male Sprague–Dawley rats weighing 220–280 g and 7 to 10 weeks old were housed individually in metabolic cages. During an adaptation period of 7 days, all rats were fed control diet containing 0.44% phosphate, 0.4% sodium, 0.41% calcium, and 0.03% magnesium (I.C.N. Pharmaceutical Co., Cleveland, Ohio). The rats were then randomly allocated to the phosphate-depleted group or the control group. The phosphate-depleted group (PD) received a diet identical to the control diet except that its phosphate content was reduced to 0.03%. Control animals continued to receive the control diet *ad libitum*. The rats had free access to deionized water.

Phosphate depletion lasted for 6 to 8 days. During this period both phosphate-depleted rats and the control animals were placed in plastic restraining cages several hours each day for adaptation. The day prior to the experiment, silastic catheters were placed in the left carotid artery and the right jugular vein and a blood sample was taken from the vein for the analysis of phosphate. Only PD animals with plasma phosphates less than 6.5 mg% were used in the study. On the morning of the experiment, the rats were anesthetized with halothane (Surital, Ayerst Laboratories, New York), a bladder catheter was placed and was connected to an extension which led out through the abdominal wall. The animals were then restrained in the plastic cages and allowed to recover from anesthesia for 1 hr.

After a priming dose of 5 μCi of [H^3]inulin (New England Nuclear, Boston, Mass.), a constant infusion of inulin was given in the jugular vein delivering 0.25 μCi/min in a solution containing 2.5% dextrose, 0.45% saline, and 5 meq/liter potassium chloride. This solution was delivered by a Harvard Infusion Pump (Model 975) at 40 μl/min throughout the experiment. After a 1-hr equilibration period, three 10-min control urine collections were obtained. Blood samples of 200 μl were collected in heparinized microcapillary tubes from the carotid catheters at the midpoint of the first and third clearance periods.

Solution of magnesium chloride in 2.5% glucose were then infused at a rate of 63 μl/min in the jugular vein, utilizing a parallel syringe. The concentration of magnesium chloride was adjusted to deliver 40, 80, 120, 160, 200, and 240 μg/min for 70–100 min at each rate. After at least 45 min of equilibration at each rate of magnesium delivery, three 10-min urine collections were obtained. Blood was withdrawn at the midpoint of the first and third clearance periods.

To determine the diffusible fraction of magnesium, eight PD and seven control rats received different rates of magnesium chloride infusion and were then bled. The heparinized blood samples were immediately centrifuged. The 2–3 ml of plasma was ultrafiltered at room temperature and without control of pH through an Amicon membrane (Centriflo, 50,000 M_r) during 15 min of centrifugation at 1000 *g*. Magnesium concentration were determined in plasma and its ultrafiltrates.

Plasma phosphate was measured by the method of Chen *et al.* (3). Plasma and urine magnesium were determined by Perkin-Elmer atomic absorption spectrophotometer

[1] Dr. Meyer is a Fellow of the Kidney Foundation of Southern California.

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served.

)3 (Perkin–Elmer Corp., Santa Ana,
H³]Inulin was counted in a Beckman
intillation counter Model LS 230
n Instrument Co., Irvine, Calif.). Fil-
ıgnesium was calculated using the
: fraction of plasma magnesium. The
ge diffusibility of magnesium was
rent from that in control animals
1.3 vs 76.6 ± 0.07%) at all levels of
ım infusion.

. The data on the body weight, the
concentrations of magnesium and
rus and glomerular filtration rates
in Table I. The PD rats weight (238
E)) was slightly but significantly (P
ess than the control animals (258 ±
: concentrations of phosphorus and
ım in plasma were significantly
' < 0.01) in the PD rats than the
(phosphorus: 5.6 ± 0.35 vs 8.1 ± 0.30
agnesium: 0.95 ± 0.05 vs 1.25 ± 0.04
r). GFR was significantly lower in
han control animals both before and
ll levels of magnesium infusion. The
in plasma calcium and magnesium
ıagnesium infusion are given in Ta-
asma calcium progressively fell dur-
ıesium infusion. This is in agreement
ilar observations in dogs (4).

ıanges in magnesium reabsorption at
of filtered magnesium produced by
ion in PD and control rats are shown
With the infusion of magnesium, its
loads increased gradually from less
) almost 14 μeq/min. In the control
ence for secretion was not detected
four- to fivefold increase in filtered
ım up to 8 μeq/min. At higher fil-
ıds produced by the higher rates of
ım infusion, only five control rats
d slight secretion of magnesium. In
in PD rats evidence for magnesium
was demonstrated at a filtered load
/min and the magnitude of the secre-
eased with the rise in filtered loads
4–5 μg/min at filtered loads of 6–12

sion. The results of the present study
at phosphate depletion in the rat is
d with hypomagnesemia, an obser-
reviously reported by us (2) and oth-
The data also demonstrate that sig-
magnesium secretion occurs in PD

TABLE I. CHANGE IN BODY WEIGHT, PLASMA LEVELS OF INORGANIC PHOSPHORUS AND MAGNESIUM, AND GLOMERULAR FILTRATION RATE IN CONTROL AND PHOSPHATE-DEPLETED RATS[a]

	n	Body weight (g)	Inorganic phosphorus (mg/dl)	Magnesium (meq/liter)	GFR (ml/min) during magnesium infusion (μg/min)					
					0	40	80	120	160	200	240
Control	7	258 ± 7	8.1 ± 0.3	1.25 ± .04	2.41 ± 0.8	2.48 ± .09	2.48 ± .09	2.28 ± .10	2.19 ± .09	2.28 ± .07	2.09 ± .08
PD	8	238 ± 4**	5.6 ± 0.3**	0.95 ± .05*	1.92 ± .11**	2.08 ± .11*	2.01 ± .10**	1.88 ± .09*	1.85 ± .08*	1.79 ± .12**	1.74 ± .10**

[a] Data are presented as mean ± SE; GFR = glomerular filtration rate; PD = phosphate depletion.
* $P < 0.05$.
** $P < 0.01$.

TABLE II. CHANGE IN THE PLASMA LEVELS OF CALCIUM AND MAGNESIUM DURING MAGNESIUM INFUSION IN CONTROL AND PHOSPHATE-DEPLETED RATS[a]

	Before magnesium infusion	During magnesium infusion (μg/min)					
Calcium mg/dl		40	80	120	160	200	240
Control (*n* 7)	9.9 ± 0.3	8.5 ± 0.3	8.0 ± 0.1	7.8 ± 0.2	7.6 ± 0.4	7.7 ± 0.4	7.3 ± 0.4
PD (*n* 8)	10.6 ± 0.55	9.5 ± 0.3	9.7 ± 0.3	9.5 ± 0.2	8.4 ± 0.3	8.3 ± 0.4	8.1 ± 0.4
Magnesium meq/liter							
Control (*n* 7)	1.25 ± 0.04	1.57 ± 0.13	2.04 ± 0.17	2.72 ± 0.21	4.03 ± 0.08	5.58 ± 0.47	7.27 ± 0.35
PD (*n* 8)	0.95 ± 0.05	1.04 ± 0.05	2.08 ± 0.19	3.25 ± 0.29	4.42 ± 0.66	6.10 ± 0.29	7.14 ± 0.32

[a] Data are presented as mean ± SE; PD = phosphate depletion.

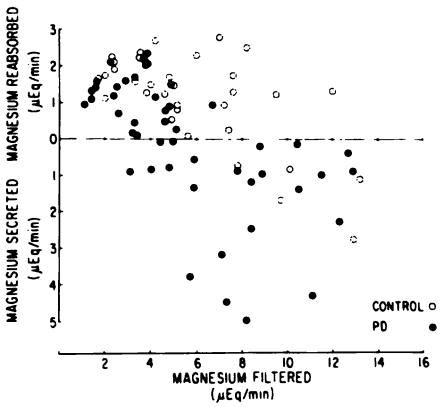

FIG. 1. The changes in the quantity of magnesium reabsorbed or secreted at various levels of filtered magnesium in control rats (○) and phosphate-depleted animals (●). Symbols below the zero line denote the magnesium secretion. Each data point is the average of two to three clearance periods obtained at the end of each level of magnesium infusion.

rats with only a twofold increase in filtered load. The magnitude of renal tubular secretion of magnesium increased as its filtered load was augmented by magnesium infusion.

In phosphate-depleted rats, the amount of magnesium reabsorbed was significantly lower and the amount secreted was significantly higher than in the control rats at any given filtered load (two-sample Hotelling t^2 test, $P < 0.01$). Furthermore, the slight magnesium secretion in the control animals occurred only after a four- to fivefold rise in filtered magnesium. These data clearly demonstrate that renal tubular magnesium secretion does occur during phosphate depletion and contributes to the magnesuria.

A great deal of controversy exists regarding magnesium secretion by the renal tubule. Averill and Heaton (5) have shown that the rat kidney secretes magnesium during magnesium loading. However, Alfredson and Walser (6) were unable to confirm this observation. Massry *et al.* (7) could not demonstrate magnesium secretion by the dog kidney during magnesium infusion and the superimposition of other factors known to decrease the tubular reabsorption of magnesium, such as extracellular fluid volume expansion (8), calcium infusion (9), and chronic DOCA administration (10). In contrast, Wen *et al.* (11) found that urinary magnesium exceeded filtered magnesium by 10–20% in some dogs receiving magnesium salts, saline, and furosemide. Thus, the available data suggest that if magnesium secretion by the nephron exists, it plays a minor role in the renal handling of magnesium.

Our data in normal rats also suggest that slight magnesium secretion may occur during high rates of magnesium infusion, an observation similar to that reported by Averill and Heaton (5). However, phosphate depletion appears to enhance the secretory process of magnesium by the kidney. It stands to reason, therefore, that at least part of the magnesuria of phosphate depletion (1, 2) is due to renal tubular secretion of magnesium. The mechanisms through which phosphate depletion enhances magnesium secretion are not as yet elucidated.

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Deleterious Effects of Leucine Administration in Endotoxin Shock (40695)

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The concept that the elaboration of circulating toxic substances during shock exacerbates the shock state has been proposed by numerous authors (1–3). Recently, we reported the isolation of one substance which exerted cardiodepression (1). We isolated (via gel filtration and cation-exchange chromatography) this substance from plasma obtained from dogs in irreversible hemorrhagic shock which we identified as L-leucine. These findings led to the following hypothesis: If leucine is a circulating toxic shock substance and if it is important in the pathogenesis of irreversible shock, then the blood levels of leucine at the time of death should be elevated in shocked animals and administration of leucine to shocked animals should hasten the onset of cardiovascular failure. In order to directly test this hypothesis, we subjected male, Sprague–Dawley rats to endotoxin shock and administered leucine, while monitoring survival times and circulating leucine levels.

Methods. a. Endotoxin shock. Two groups of male rats (350–375 g) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). The femoral artery was cannulated and 1.0 mg/100 g *Salmonella enteritidis* endotoxin (Difco, Batch 649204) was injected over 1 min. Pilot studies revealed that this dose of endotoxin was approximately an LD_{90}. Immediately after endotoxin injection, either 0.153 *M* leucine or isoleucine was infused at a dose of 7.6 mmole/kg, or the equivalent volume of Krebs–Henseleit vehicle was infused through the femoral artery catheter. These solutions were infused at 0.33 ml/min and took approximately 35 min for each animal. This long infusion time and high infusion volume was necessitated by the low water solubility of leucine. In the first group, each animal had three blood samples (0.3 ml each) drawn at the following times: (i) prior to endotoxin infusion; (ii) after amino acid or vehicle infusion; and (iii) at death. After endotoxin and either leucine, isoleucine, or KH infusion, each animal was observed and the time between the end of infusion and death (defined as the spontaneous cessation of heart beat via manual palpitation) was recorded. Infusion of 7.6 mmole/kg leucine into nonshocked rats was also performed. In the second group, blood samples (2 ml) were taken at ½, 2, or 3 hr after the end of leucine or KH infusion, and the animal was terminated.

b. Blood sample analysis. The collected blood samples from each animal were allowed to clot and centrifuged and the serum was collected. Each serum sample was deproteinized by the mixing of 100 μl 10% trichloroacetic acid containing 12.5 nmole of norleucine as an internal standard to 100 μl serum. After centrifugation, 100 μl of deproteinized serum was applied to an Aminex A-4 cation-exchange column (0.9 × 50 cm) and eluted with Pico buffer B (Pierce Chemical Co.) at 50° and a flow rate of 60 ml/hr. In this system, all of the acidic amino acids elute with the buffer front and methionine, isoleucine, leucine, and norleucine elute at 51, 58, 64, and 70 min, respectively. For the detection system we used an automated orthophthaldyhyde (OPA) reaction system (4) where the reagent consisted of 0.08% OPA in 0.4 *M* potassium borate, pH 10.4, which was mixed by pumping at 48 ml/hr with the column effluent in a 0.5-mm-i.d. × 3-m-length Teflon coil. Fluorescence was measured in a continuous-flow fluorometer (Laboratory Data Control) and recorded at 6 in./hr on a 10-in. strip chart recorder. Peak integration was performed by manual triangulation and the data were expressed in μ*M* concentration. After each analysis the column was regenerated for 15 min with 0.7 *N* NaOH containing 0.2% w/v ethylenediamine tetraacetic acid

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odium salt and 0.2% v/v of a 30% Brij
ıtlas Chem. Ind.) solution in order to
nt loss of resolution due to peptide ab-
ion on the ion-exchange resin.

Data analysis. The difference between
me of death for leucine, isoleucine- and
ıdministered rats and the serum amino
concentrations at analogous times were
ıared using the Student's *t* test for un-
d data to determine significance. The
ges in serum amino acid concentrations
ch group at sequential times were com-
l using the paired Student's *t* test. Cor-
on of the time to death and each serum
o acid concentration was performed us-
near regression analysis. Significance in
sts were assumed to be reached at $P <$

sults. The data derived from the first
) of endotoxin-shocked rats is presented
ble I. The infusion of leucine into endo-
-shocked rats ($N = 8$) accelerated the
: syndrome as evidenced by the signifi-
y lower time to death in the leucine
als ($P < 0.01$ compared to KH-infused
ted rats) ($N = 9$). The isoleucine-admin-
d shock rats ($N = 9$) tended to expire
er than the KH-infused shock rats ($P =$
. Infusion of leucine or isoleucine at 7.6
le/kg body wt concentrations in non-
ted rats had no apparent effect, i.e., these
als were 24-hr survivors. Measuring the
n concentration of leucine and isoleucine
led that after endotoxin administration
KH infusion, both leucine and isoleucine
entrations were elevated at death ($N =$
ucine: from 140 ± 9 to 351 ± 25 μM, (P
)1); isoleucine: 104 ± 6 to 176 ± 4 μM
0.01), mean ± SEM). In the isoleucine-
ed animals the serum isoleucine concen-
trations at the time of death were significantly elevated ($P < 0.01$). However, in these animals, serum leucine was also elevated compared to KH-infused shocked rats at time of death (507 ± 27 vs 351 ± 25 μM, respectively, $P < 0.05$). In contrast, the isoleucine concentrations at the time of death in the leucine-administered animals were not different from the isoleucine concentration in shocked rats given KH (176 ± 4 vs 174 ± 18 μM, respectively). The serum leucine concentrations at the time of death in the leucine-infused rats were significantly higher than both other groups ($P < 0.01$).

The terminal serum leucine concentrations were elevated in all groups compared to preshock levels. There was an inverse correlation between blood leucine concentration at time of death and time to death ($r = 0.415$, $P = 0.02$). The data appears to be curvilinear rather than linear which may account for the low correlation coefficient. The observations that terminal serum leucine concentrations in the isoleucine-infused, endotoxin-shocked animals were elevated compared to those of KH-infused rats and that the isoleucine-infused animals also died earlier than the KH-infused rats was unanticipated.

The serum leucine concentration of the second group of rats is presented in Table II. Following KH infusion in the sham-shocked rats, leucine levels did not change significantly. Following leucine infusion in the sham rats, serum leucine concentrations were markedly elevated and they remained so through the 3-hr observation. The KH-infused endotoxin shock group demonstrated a significant transient elevation in serum leucine at ½ hr. The endotoxin-shocked, leucine-infused rats had a significantly higher serum

ABLE I. SERUM LEUCINE AND ISOLEUCINE CONCENTRATIONS FOLLOWING ENDOTOXIN ADMINISTRATION

		Time to death	Leucine (μM)			Isoleucine (μM)		
ıfusate	*N*	(min)	A	B	C	A	B	C
Henseleit	9	274 ± 24	140 ± 9	212 ± 10[a]	351 ± 25[a]	104 ± 6	122 ± 3	176 ± 4[a]
:ine	9	201 ± 26[b]	145 ± 7	[c]	507 ± 27[a, d]	104 ± 4	14075 ± 1289	2020 ± 824[a, d]
ıe	8	185 ± 10[d]	139 ± 8	11343 ± 1138	839 ± 89[a, d]	102 ± 4	—[e]	174 ± 18[a]
							—	

ata reported as mean ± 1 SEM. Sample A prior to endotoxin, B after infusion, C at death. [a] $P < 0.01$ compared
ple A, paired *t* test.
= 0.06 compared to KH, group *t* test.
ɔt measurable due to high isoleucine concentration.
< 0.05 compared to KH, group *t* test.
ɔt measurable due to high leucine concentration.

TABLE II. SERUM LEUCINE CONCENTRATION FOLLOWING ENDOTOXIN OR VEHICLE INJECTION AND LEUCINE OR KREBS-HENSELEIT INFUSION

Group	½ hr	2 hr	3 hr
Sham-KH	190 ± 30 (5)	158 ± 12 (5)	165 ± 5 (5)
Sham-Leu	4973 ± 226 (4)	842 ± 101 (5)	456 ± 57 (6)
Endo-KH	336 ± 31 (6)	134 ± 7 (5)	152 ± 11 (6)
Endo-Leu	6589 ± 167[a] (6)	1510 ± 270[b]	843 ± 176[b] (5)

[a] All values ± SE (N) μM leucine. Time after end of leucine or KH infusion. Value prior to endotoxin or sham injection is 142 ± 5 M. $P < .01$ compared to Sham-leu.

[b] $P < .05$ compared to Sham-leu.

[c] $P < 0.01$ compared to Sham-KH.

leucine concentration at all times following infusion than did the sham-shocked, leucine-infused rats indicating that endotoxin shock inhibited the catabolism of the exogenous leucine.

Discussion. The experiments reported in this study were based on our observations that leucine may be a circulating toxic shock substance (1, 3). We tested the hypothesis that if leucine was a toxic shock substance, then the exogenous administration of leucine to a shocked animal should accelerate the shock syndrome and serum leucine concentrations should be elevated in the vehicle-infused shocked animals. The dose of leucine (or isoleucine, used as an amino acid control) chosen for infusion was calculated to raise serum leucine at the end of infusion to approximately 7.6 mM, a level which exerted significant depressant activity in the feline right ventricular papillary muscle bioassay as previously reported (1, 3). We rationalized that if it did not accelerate the shock syndrome at this high concentration, then it probably would not have any *in vivo* significance. Cardiac function was not measured in these animals because of a complex interpretive problem. *A priori,* since all of these animals will exhibit cardiac failure after endotoxin administration (LD_{90}), it would be impossible to determine if the rate of the development of cardiac hypodynamics was faster than the rate of the development of cardiovascular collapse in the leucine-infused animals compared to the KH-infused controls.

The findings of this study indicate that elevation of serum leucine may be a contributing factor in the development of the shock syndrome. The administration of these large amounts of exogenous leucine did significantly accelerate the endotoxin shock syndrome. An elevation of serum leucine by a factor of 2.5 in the KH-infused, endotoxin-shocked rats indicated that leucine may indeed exert a pathophysiological action *in vivo.* Although the exact mechanism for the shock-accelerating action of leucine was not approached in this study, the observation that leucine did not induce shock in nonshocked animals suggests that leucine acts synergistically with another shock product and/or the shock syndrome induces a dysfunction of leucine metabolism allowing for its serum accumulation. A specific shock-accelerating role for leucine was suggested by observation of elevated sera leucine concentrations in the isoleucine-infused group which was associated with an earlier time of death for these animals. Since the converse (elevated isoleucine levels in the leucine-infused rats) was not observed, this suggests a specific role for leucine rather than a nonspecific effect of amino acids. The apparent shock-accelerating action of leucine may be magnified by a dysfunction of leucine catabolism following endotoxin. We observed significantly higher serum leucine concentrations after leucine infusion in the endotoxin-shocked rats than in the sham animals.

Previous investigations have reported similar plasma or serum elevations in leucine concentrations following traumatic injury or clinical shock episodes (5, 6). Levenson *et al.* (5) reported that plasma leucine levels do indeed rise dramatically during the initial response to traumatic injury caused by battle casualties. They reported increases in the range of two to five times control values; the concentrations reported were in the concentration range which we found to be cardiodepressant *in vitro* (3). Border *et al.* (6) have also reported elevated plasma leucine levels of traumatically injured patients. They reported that leucine, along with many other amino acids, ketones, and triglycerides begin at a low level, reach maximum values at 5–10 days, and drop by 21 days in patients successfully treated for multiple organ system failure induced by traumatic injury via gunshot wound, automobile, or industrial accidents.

Summary. We have recently reported the isolation and identification of leucine from the plasma of shocked animals which exerted significant cardiodepressant activity *in vitro.* This study tested the hypothesis that if leucine is a circulating-factor *in vivo* and if this has any significant deleterious effects in the shocked animal, then the exogenous administration of leucine should accelerate the shock syndrome. We infused leucine, isoleucine (7.6 mmole/kg), or KH into endotoxin-shocked rats and measured survival times and blood concentrations of leucine and isoleucine. Following endotoxin administration, the leucine-administered rats died significantly sooner ($P < 0.01$) than the KH-infused animals; the isoleucine animals also died sooner ($P = 0.06$). Terminal leucine concentrations were elevated in all animals following endotoxin administration compared to preshock concentrations. The terminal serum leucine concentration in the leucine- and isoleucine-infused groups was significantly higher ($P < 0.06$) than the KH-infused group. In contrast, terminal serum isoleucine concentrations were significantly elevated only in the isoleucine-infused group. These data suggest that the elevated serum leucine concentration may contribute to the development of irreversible shock.

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Altered Erythropoiesis during the Course of Virulent Murine Malaria (40696)[1]

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Anemia is a major pathologic consequence of malaria in humans (1–3) and in rodents (4–7). There is abundant evidence which supports the thesis that the anemia associated with malarial infection is attributed to the destruction of parasitized and nonparasitized erythrocytes in concert with immune and phagocytic mechanisms (6–10). However, since the maintenance of normal circulating erythrocyte levels in mammals is governed by the precise homeostatic balance between erythropoiesis and erythrocyte destruction (11–13), it is conceivable that impaired erythropoiesis may also contribute to the anemic status of malaria-infected organisms. Supportive evidence has shown that during acute primary *P. vivax* and *P. falciparum* infections in humans, the proportions of marrow erythroid precursors (i.e., pronormoblasts through orthochromatophilic normoblasts) are depressed and impaired in their ability to incorporate radioiron *in vitro* (14, 15). In contrast, however, it has been suggested from semiquantitative data, that erythropoiesis in mice is enhanced at medullary and ectopic sites in response to the attendant anemia and hypoxemia of malarial infection (16). Nevertheless, it is clear that erythropoiesis does not keep pace with the enhanced rate of erythrocyte destruction and malarial mice quickly succumb, in part, to the deleterious consequences of severe uncompensated anemia (4–7). The present study was undertaken to elucidate the questionable relationship between the erythropoietic activity and anemia of mice during the course of virulent malarial infection.

Materials and methods. Virgin female CD-1 mice (Charles River Labs.), 14–16 weeks of age, were employed throughout. Malarial infection was transferred by a single intraperitoneal inoculum containing 15.0×10^6 erythrocytes parasitized with *Plasmodium berghei* (NK/65 strain). On Days 7, 14, and 21, control and infected mice were anesthetized with ether and sacrificed by cardiac puncture. Measurements on circulating blood, marrow, and spleen were performed as follows:

Blood measurements. Erythrocyte enumerations were obtained electronically with a Coulter Counter (Model Z_{B1}, Coulter Electronics, Inc.). The percentage hematocrits were determined by standard techniques. Hemoglobin was measured in accord with the cyanmethemoglobin procedure (17). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) of blood samples were calculated by standard procedures (18). The percentage reticulocytes were determined from blood stained with new methylene blue (19) and % parasitemias were scored from methanol-fixed blood smears stained with Giemsa solution. Based on individual erythrocyte counts, the percentage reticulocytes and parasitemias were converted to absolute numbers/mm^3 of blood.

Medullary measurements. The numbers of nucleated cells/tibia were determined by flushing the bone with cold TC medium 199 (Difco Labs) and enumerating the monocellular suspension by hemocytometer subsequent to lysis of mature erythrocytes with 3% glacial acetic acid. The percentage of erythroid precursors/femur were scored from methanol-fixed paint-brush smears, stained with Wright's and Giemsa solutions (20). The numbers of erythroid precursors/tibia were calculated from the tibial nucleated cellularity and the proportion of femoral erythroid precursors.

Splenic measurements. The numbers of splenic nucleated cells were determined by hemocytometer, subsequent to splenic mincing in cold TC medium 199 and sequential

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ge through 19- to 23-gauge needles. The throid precursors/spleen were scored methanol-fixed smears stained with it's and Giemsa solutions (20). The ers of splenic erythroid precursors were ated from the splenic nucleated cellu- and the proportion of erythroid precur-

parameters were expressed as the daily mean ± 1 standard error. Based on the rametric Mann–Whitney U test (21), $P < 0.05$ were considered to be statistiignificant.

ults. Table I demonstrates the erythroid status of CD-1 mice during the course of malarial (*P. berghei*) infection. It is clear that infected mice become progressively anemic, with circulating erythrocyte, hematocrit, and hemoglobin levels declining to approximately 20% of control values ($P < 0.05$) on Day 21. Specifically, the anemia was characterized by a 34% increase in mean corpuscular volume ($P < 0.05$), a 16% elevation in mean corpuscular hemoglobin ($P < 0.05$), and a 14% decrease in mean corpuscular hemoglobin concentration ($P < 0.05$) on Day 21. A slight 1.5-fold peak reticulocytosis was evidenced on Day 7, which declined thereafter. Tibial

TABLE 1. ERYTHROID STATUS[a] OF CONTROL AND MALARIAL MICE[b]

Parameters	Days of infection		
	7	14	21
ted erythrocytes ($\times 10^5/mm^3$)			
trols	0 (20)	0 (20)	0 (12)
rimentals	3.5 ± 0.3 (20)[c]	6.6 ± 0.6 (20)[c]	5.2 ± 0.5 (17)[c]
cytes ($\times 10^6/mm^3$)			
trols	8.5 ± 0.2 (20)	8.3 ± 0.3 (20)	8.2 ± 0.2 (12)
rimentals	5.6 ± 0.3 (20)[c]	2.6 ± 0.2 (20)[c]	1.4 ± 0.1 (17)[c]
crit (%)			
trols	44.4 ± 0.9 (20)	43.9 ± 1.0 (20)	43.3 ± 0.6 (12)
rimentals	29.8 ± 1.2 (20)[c]	16.8 ± 1.0 (20)[c]	9.9 ± 0.8 (17)[c]
obin (g/dl)			
trols	14.4 ± 0.4 (20)	15.2 ± 0.5 (20)	14.7 ± 0.3 (12)
riments	9.6 ± 0.4 (20)[c]	4.8 ± 0.4 (20)[c]	2.9 ± 0.1 (17)[c]
orp. volume (μm^3)			
trols	52.2 ± 0.6 (20)	52.9 ± 1.4 (20)	52.8 ± 2.3 (12)
rimentals	53.2 ± 1.3 (20)	64.6 ± 4.5 (20)[c]	70.7 ± 4.3 (17)[c]
orp. hemoglobin (pg)			
trols	16.9 ± 0.3 (20)	18.3 ± 0.5 (20)	17.9 ± 0.8 (12)
rimentals	17.1 ± 0.6 (20)	18.5 ± 1.0 (20)	20.7 ± 1.3 (17)[c]
orp. hemoglobin conc. (%)			
trols	32.4 ± 0.4 (20)	34.6 ± 0.5 (20)	33.9 ± 0.4 (12)
rimentals	32.2 ± 0.6 (20)	28.6 ± 0.7 (20)[c]	29.3 ± 1.2 (17)[c]
ocytes ($\times 10^5/mm^3$)			
trols	1.7 ± 0.2 (19)	2.0 ± 0.2 (20)	1.6 ± 0.3 (11)
rimentals	2.6 ± 0.3 (20)	2.2 ± 0.3 (20)	2.0 ± 0.3 (16)
ed cells/tibia ($\times 10^6$)			
trols	12.8 ± 0.9 (20)	13.0 ± 0.9 (20)	14.9 ± 1.0 (12)
rimentals	10.9 ± 0.9 (20)[c]	10.5 ± 0.6 (20)[c]	10.7 ± 0.8 (17)[c]
id precursors/tibia ($\times 10^6$)			
trols	2.3 ± 0.2 (19)	2.3 ± 0.2 (20)	2.4 ± 0.2 (12)
rimentals	1.7 ± 0.3 (19)[c]	1.8 ± 0.2 (18)[c]	1.8 ± 0.2 (17)[c]
ed cells/spleen ($\times 10^8$)			
trols	2.2 ± 0.2 (20)	2.5 ± 0.2 (20)	2.6 ± 0.2 (12)
rimentals	4.3 ± 0.8 (18)[c]	10.4 ± 1.0 (20)[c]	9.5 ± 0.9 (15)[c]
id precursors/spleen ($\times 10^7$)			
trols	1.7 ± 0.2 (19)	2.2 ± 0.3 (19)	1.9 ± 0.4 (12)
rimentals	8.7 ± 1.1 (17)[c]	40.7 ± 5.6 (20)[c]	37.2 ± 6.4 (15)[c]

lues represent the daily group mean ± 1 standard error and reflect the combined data from three separate ients. Values in parentheses refer to the total numbers of mice per group. Statistical significance between ntrol and experimental groups was assessed by the nonoparametric Mann–Whitney U test.

e were infected on Day 0 with an intraperitoneal inoculum containing 15.0×10^6 parasitized (*P. berghei*) ytes.

nificantly different from daily control values ($P < 0.05$).

nucleated cellularity was depressed to approximately 85% ($P < 0.05$) of control levels on Day 7 and further declined to 72% ($P < 0.05$) on Day 21. The numbers of tibial erythroid precursors remained at approximately 74% ($P < 0.05$) of control values throughout the course of infection. In contrast, the numbers of splenic nucleated cells were elevated 2-fold ($P < 0.05$) on Day 7 and increased approximately 4-fold ($P < 0.05$) on Days 14 and 21; at which time nearly 20-fold increases in erythroid precursor levels ($P < 0.05$) were apparent.

Discussion. In consideration of the severe anemia associated with virulent rodent malaria, much attention has been directed toward an understanding of processes associated with the destruction of parasitized and nonparasitized erythrocytes (6–10), but alternative possibilities remain ill defined. The present study was undertaken to further characterize the erythropoietic status of mice during the course of *P. berghei* infection in an attempt to determine if erythropoietic impairment contributed to their anemic status.

Coincident with the intense parasitemia which peaked on Day 14, infected mice became progressively anemic, with circulating erythrocyte, hematocrit, and hemoglobin values declining to approximately 20% of control levels on Day 21. These data agree with previous reports (4–7). Specifically, however, we noted that the anemia of malarial mice was characterized by a 34% increase in mean corpuscular volume, a 16% elevation in mean corpuscular hemoglobin, and a 14% decrease in mean corpuscular hemoglobin concentration on Day 21 of infection.

Malarial mice developed a peak 1.5-fold reticulocytosis on Day 7, which declined thereafter. Accordingly, the reticulocyte response reported herein was meager in comparison to what one might expect in other situations characterized by similar degrees of anemia (22). While these data lend support to the suggestion that impaired erythropoiesis contributes to the anemic status of malarial mice, based on the reticulocyte response alone, this conclusion must be viewed with caution since *P. berghei* parasites have a definite predilection for reticulocytes, as opposed to the more mature erythrocytic element (6, 10). Furthermore, the reticulocytosis may, in fact, be an underestimation of the true erythropoietic response by virtue of a shortened cell lifespan, as reported in malaria (6–10).

In view of the problems associated with assessing the dynamics of erythropoiesis in malaria from circulating parameters, we chose to monitor the erythroid responses at bone marrow and splenic sites. On Day 7 the numbers of tibial nucleated cells and erythroid precursors in infected mice were depressed to 85 and 74% of control values, respectively. Tibial nucleated cellularity declined further to 72% of control levels on Day 21, while the numbers of erythroid precursors remained approximately 26% depressed throughout the course of infection. These data are in agreement with previous studies which have shown that in humans, during acute primary *P. vivax* and *P. falciparum* infections, marrow erythroid precursors are likewise decreased (14, 15). However, it was not feasible in their human study to monitor the total marrow erythroid content/bone. Hence, by comparison, our data perhaps affords more conviction in view of the significant depression in absolute numbers of erythroid precursors/tibia throughout the course of murine malaria. Marrow depression/inhibition has been suggested in the past during acute malarial infections (18, 23, 24).

The depressed medullary erythroid findings we observed are not in agreement with a previous study (16) which reported that erythropoiesis in malarial mice is enhanced at medullary sites. In that study, Singer employed histologic sections of bone marrow and clearly stated the lack of strict quantitation thereof. In our investigation, however, we circumvented this problem by preparing a monocellular suspension from which the absolute numbers of erythroid precursors were determined. Hence, our manipulation may have permitted a better representation of the medullary erythroid capacity of *P. berghei*-infected mice, and negates the possibility that erythropoiesis might be expanded into normally nonerythropoietic portions of the marrow, since the lower one-third of the tibia is such an area. In any case, the depressed medullary erythroid response which we report during murine malaria, may perhaps be likened to that seen in situations involving other infections (25).

In contrast to the depressed medullary response noted in *P. berghei*-infected mice, the

numbers of splenic nucleated cells were elevated 2-fold on Day 7 and rose to approximately 4-fold on Days 14 and 21, at which time nearly 20-fold increases in erythroid precursor levels were apparent. These data are in agreement with the qualitative observations made by Singer (16). Apparently normal mice, subjected to intense erythropoietic challenge, have the capacity to undergo erythropoietic expansion at both medullary and splenic sites. In the past, we (22) have, for example, shown that subsequent to intense phenylhydrazine-induced hemolytic anemia, erythropoiesis is increased approximately 2-fold in medullary sites and 50-fold in the spleen. Conversely, in *P. berghei*-infected mice, medullary erythropoiesis was significantly depressed and splenic erythropoiesis, although expanded, did not increase to the extent expected. Furthermore, since the murine spleen during normalcy usually accounts for approximately 20% of total red cell production (26), the contrasting increased splenic and decreased medullary erythropoiesis during malaria, could perhaps account for the meager reticulocytosis, as reported herein.

Although the importance of chemical and cellular modulators of erythropoiesis is well recognized (27–29), the specific mechanisms responsible for impaired erythropoiesis in malarial mice are presently unknown, but may perhaps relate to: (a) hemopoietic stem cell (i.e., CFU-s, BFU-e, and/or CFU-e) deficits, and/or (b) committed erythroid stem cell (i.e., BFU-e and/or CFU-e) resistance to erythropoietin, and/or (c) the existence of ineffective erythropoiesis. Plans to elucidate these possibilities are in progress.

Summary. Peripheral, medullary, and splenic erythroid parameters were quantitatively assessed in mice during the course of virulent malarial (*P. berghei*) infection. Mice became progressively anemic, with circulating erythrocytes, hematocrit, and hemoglobin levels declining to approximately 20% of control by Day 21. The anemia was further characterized by a 34% increase in mean corpuscular volume, a 16% elevation in mean corpuscular hemoglobin, and a 14% decrease in mean corpuscular hemoglobin concentration by Day 21. A meager 1.5-fold reticulocytosis was noted on Day 7, which declined thereafter. Tibial nucleated cellularity was depressed to 85 and 72% of control values on Days 7 and 21, respectively, while erythroid precursor numbers remained at 74% of control levels throughout the course of infection. In contrast, splenic nucleated cells were elevated 2-fold on Day 7 and increased approximately 4-fold on Days 14 and 21. Although nearly 20-fold elevations in erythroid precursor levels were apparent, these increases were below what one might expect in noninfected mice with a similar degree of anemia.

These data support the contention that the erythropoietic capability of mice infected with malaria is impaired. Accordingly, it is suggested that this situation contributes to the severe anemic status of malarial mice, in addition to the well-documented mechanisms leading to the destruction of parasitized and nonparasitized erythrocytes.

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The Effects of Captopril, Propranolol, and Indomethacin on Blood Pressure and Plasma Renin Activity in Spontaneously Hypertensive and Normotensive Rats (40697)

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The mechanisms by which renin release may be altered have been the subject of numerous reports and have been reviewed by several authors (1–5). In the past, most studies centered around the control of renin release by the intrarenal receptors in the afferent arteriole and macula densa, adrenergic receptors, and a few humoral agents, such as angiotensin II and antidiuretic hormone. However, it has become obvious in recent years that prostaglandins may also play an important role in controlling renin release. Prostaglandins such as PGE_1, PGE_2, PGA_1, and prostacyclin (PGI_2) cause renin release both *in vitro* and *in vivo* (6–10). Similarly, the precursors to the stable prostaglandins, arachidonic acid, and the prostaglandin endoperoxides also cause an increase in renin release (11–14). Moreover, inhibition of prostaglandin synthesis reduces resting plasma renin activity (PRA) and prevents increases in renin secretion caused by arachidonic acid as well as the increase caused by changes in posture and the diuretic furosemide (15–22).

Captopril (SQ 14,225, D-3-mercapto-2-methylpropanoyl-L-proline) is an orally effective inhibitor of angiotensin-converting enzyme which reduces blood pressure in normal renin as well as high renin animal models of hypertension (23–28). Similarly, captopril is an effective antihypertensive agent in low, normal, and high renin essential hypertension as well as in renal hypertension in man (29–31). In all of the studies in which it was measured, PRA increased substantially after captopril administration in both normal and hypertensive animals and man (24, 25, 29–32). The mechanism(s) by which captopril raises plasma renin activity has not been examined although many possibilities such as reflex sympathetic activation, interruption of a negative feedback system, and increased renal prostaglandin formation could very well play a role.

The purpose of this study was to examine the effects of captopril alone and in combination with either the β-receptor antagonist, propranolol, or the cyclooxygenase inhibitor, indomethacin, on blood pressure and PRA in normotensive rats and in spontaneously hypertensive rats.

Materials and methods. Ten- to fourteen-week-old male normotensive rats of the Wistar–Kyoto strain (WKY) and spontaneously hypertensive rats of the Okamoto and Aoki strain (SHR) were obtained from Taconic Farms, Germantown, N.Y. and were placed on a normal rat chow diet with water *ad libitum*. All drugs were administered by gavage.

Captopril was synthesized at the Squibb Institute for Medical Research. Indomethacin was kindly donated by Merck, Sharpe and Dohme, propranolol HCl was kindly donated by Ayerst Laboratories, and arachidonic acid was purchased from Sigma Chemical Company.

Statistics were calculated using an unpaired Student's t test. A value of $P < 0.05$ was considered to be significant.

Blood pressure measurements. Approximately 18 days before each of the dosage intervals described below, indwelling abdominal aortic cannulae were implanted in rats anesthetized with pentobarbital sodium and, after the 18-day recovery period, direct mean arterial blood pressures in conscious rats were recorded by the method of Laffan *et al.* (25).

Plasma renin activity. On the day of blood pressure measurement, 1 hr after any drug treatment, 0.5 ml of blood from the implanted aortic cannula of all rats was collected into a tube containing 50 μl of 0.25 m*M* EDTA, pH 7. Plasma was prepared by centrifuging the samples at 2500 g for 30 min at 4°. Two hundred microliters of plasma was placed in a 400-μl polypropylene tube, tightly capped, and stored in an ultra-low temperature

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freezer (−70°) until analyzed. PRA values were usually determined within 3 weeks of storage.

PRA values, expressed in terms of nanograms of Angiotensin I (Asp^1–Ile^5–angiotensin I) generated per milliliter of plasma per hour of incubation at 37°, were determined using the Squibb Angiotensin I Immunotope Kit. The pH of the plasma was adjusted to 6.5 which is the pH optimum for PRA in rats.

Results. Effects of captopril, indomethacin, and propranolol on mean blood pressure (MBP). Treatment of spontaneously hypertensive rats (SHR) with captopril (30 mg/kg p.o. daily for 3 days) resulted in a significant reduction in MBP (Table I). Similar, though smaller, reductions in MBP were observed after propranolol (30 mg/kg p.o. daily for 3 days). The combination of captopril and propranolol administered together also resulted in significant reductions in MBP which were not significantly different from those observed with either captopril or propranolol given alone (Table I). Propranolol treatment resulted in complete blockade of the normally observed tachycardia (54.3 ± 2.6 beats/min) caused by isoproterenol administration, 1 μg/kg i.v.

TABLE I. EFFECTS OF CAPTOPRIL AND PROPRANOLOL (BOTH AT 30 mg/kg p.o. DAILY FOR 3 DAYS), ALONE AND IN COMBINATION, ON MEAN BLOOD PRESSURE (MBP, mm Hg)[a] OF SPONTANEOUSLY HYPERTENSIVE RATS (SHR) AND NORMOTENSIVE RATS (NTR)

	Control MBP	N	Captopril	Propranolol	Captopril + propranolol
SHR	183.6 ±3.4	7	136.7 ±6.5 $P < 0.001$		
	188.0 ±3.0	10		153.6 ±5.6 $P < 0.001$	
	178.0 ±2.3	7			150.6 ±7.7 $P < 0.005$
NTR	129.5 ±3.4	10	110.3 ±5.1 $P < 0.02$		
	130.7 ±2.1	6		105.8 ±7.0 $P < 0.01$	
	128.6 ±2.8	7			108.0 ±5.3 $P < 0.02$

[a] Values shown after drugs are the maximum changes observed over a 3-hr observation period on the third day of dosing.

TABLE II. EFFECTS OF CAPTOPRIL (30 mg/kg p.o DAILY FOR 3 DAYS) AND INDOMETHACIN (2.5 mg/kg p.o. DAILY FOR 3 DAYS), ALONE AND IN COMBINATION, ON MEAN BLOOD PRESSURE (MBP, mm Hg)[a] OF SPONTANEOUSLY HYPERTENSIVE (SHR) AND NORMOTENSIVE RATS (NTR)

	Control MBP	N	Captopril	Indomethacin	Captopril + indomethacin
SHR	169.0 ±2.0	10	145.3 ±4.0 $P < 0.001$		
	175.9 ±3.6	9		162.7 ±6.7	
	183.4 ±5.1	10			138.6 ±5.4 $P < 0.01$
NTR	118.9 ±2.1	9	110.7 ±4.3 $P < 0.05$		
	122.4 ±1.5	10		117.8 ±2.9	
	116.5 ±1.7	4			100.8 ±2.5 $P < 0.05$

[a] Values shown after drugs are the maximum changes observed over a 3-hr observation period on the third day of dosing.

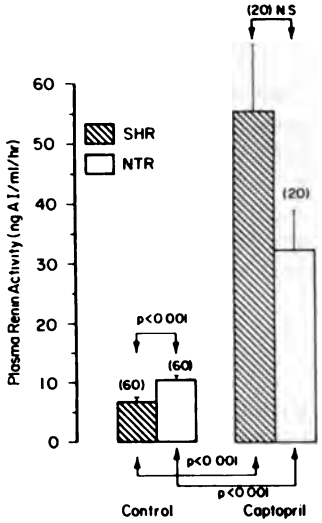
Qualitatively similar results were observed in normotensive rats (Table I).

In other SHR, captopril (30 mg/kg p.o. daily) administered for 3 days once again caused a significant reduction in MBP (Table II). Treatment with indomethacin (2.5 mg/kg p.o. daily for 3 days) had no effect on MBP by itself nor did it affect the reduction in MBP caused by captopril (Table II).

Once again, qualitatively similar results were observed in normotensive rats (Table II).

Effects of captopril, indomethacin, and propranolol on plasma renin activity (PRA). PRA of untreated SHR were significantly lower than those of NTR (Fig. 1). Administration of captopril (30 mg/kg p.o. daily for 3 days) caused significant, marked elevation of PRA in both SHR and NTR. Although the maximum absolute PRA values after captopril in SHR and NTR were not significantly different from each other (Fig. 1), the mean value of the differences was (48.03 vs 22.7 for SHR and NTR, respectively; $P < 0.05$).

Indomethacin (2.5 mg/kg p.o. daily for 3 days) significantly reduced PRA in both SHR and NTR (Fig. 2). Whereas indomethacin had no significant effect on changes in PRA caused by captopril in SHR, it caused a mod-



G. 1. Plasma renin activity (ng angiotensin I/ml/ ı conscious spontaneously hypertensive (SHR) and ır-Kyoto normotensive (NTR) rats before and after ›pril (30 mg/kg p.o.). Figures in parentheses indicate ›ers of rats/group.

though insignificant, inhibition of renin ase in NTR (Fig. 2, cf. Fig. 1). The dose ıdomethacin used caused a 61.1% inhibi- of the depressor response (64.2 ± 9.8 mm 5.0 ± 5.5 mmHg, $P < 0.001$) to arachi- ic acid (0.3 mg/kg i.v.) in other SHR. her doses of indomethacin were found to oxic.

ropranolol (30 mg/kg p.o. daily for 3 s) reduced PRA in both SHR and NTR ;. 3). Propranolol also prevented any sig- :ant rise in PRA in SHR dosed with topril whereas the effect in NTR was not ificantly reduced (Fig. 3, cf. Fig. 1).

Discussion. The present study demon- tes, as have others (32–34), that adult R have significantly lower plasma renin vities (PRA) than their normotensive ge- c counterparts. We have also confirmed ability of captopril to increase plasma n activity and to decrease blood pressure HR (25) and have further demonstrated ncrease of PRA in WKY–NTR rats after topril, which is similar to that of the ague–Dawley strain (26).

oth indomethacin and propranolol sed significant reductions in plasma renin vity in both SHR and NTR before the inistration of captopril suggesting that n release in these animals is under tonic control by both β-receptors and prostaglandins as has been previously suggested (see Introduction for references). In SHR, propranolol markedly decreased, whereas indomethacin was without effect on, the increase in PRA caused by captopril administration. However, in NTR, neither propranolol nor indomethacin significantly inhibited the effects of captopril on PRA. These results sug-

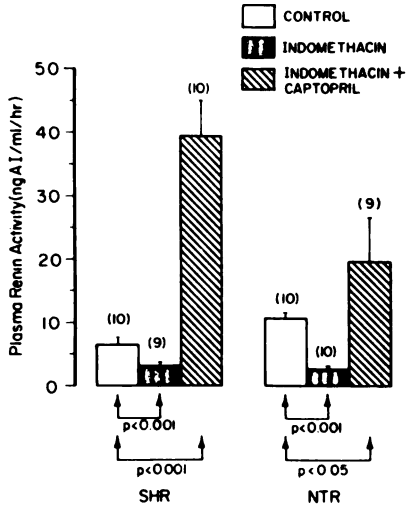


FIG. 2. Plasma renin activity (ng angiotensin I/ml/hr) in conscious SHR and NTR before and after either indomethacin alone (2.5 mg/kg p.o.) or indomethacin + captopril (30 mg/kg p.o.). Figures in parentheses indicate numbers of rats/group.

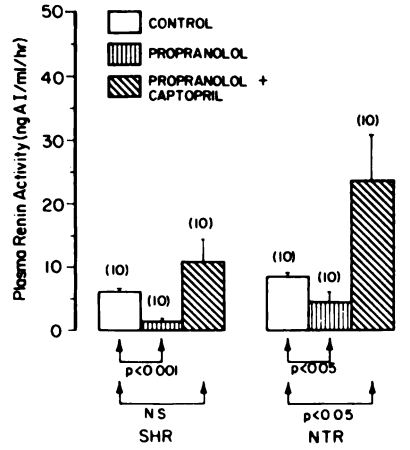


FIG. 3. Plasma renin activity (ng angiotensin I/ml/hr) in conscious SHR and NTR before and after either propranolol (30 mg/kg p.o.) or propranolol + captopril (30 mg/kg p.o.). Figures in parentheses indicate numbers of rats/group.

gest that, at least in SHR, the increase in PRA caused by captopril administration is mediated mainly by activation of renal β-receptors. Furthermore, since propranolol had little effect on either blood pressure or the increase in PRA in NTR, this activation was probably a result of a compensatory reflex sympathetic activation caused by the reduction in blood pressure by captopril. It should be noted that propranolol also prevents the increase in plasma renin activity caused by agents such as saralasin which act by interrupting the short-loop intrarenal feedback mechanism (35). Thus, it is conceivable that propranolol acts distal to the angiotensin II receptor in this short loop mechanism after captopril administration which reduces angiotensin II formation.

Interestingly, the addition of propranolol to captopril therapy in SHR had no significant effect on the reduction in blood pressure by captopril indicating the high plasma renin activity and angiotensin I level did not substantially override the inhibition of the angiotensin-converting enzyme in these animals.

Indomethacin had no significant inhibitory effect on either the antihypertensive action of captopril in SHR or on the increase in plasma renin activity in both SHR and NTR. Thus, prostaglandin formation does not seem to play a significant role in the ability of captopril to decrease blood pressure in SHR or in raising renin levels. However, indomethacin did significantly decrease resting PRA values in both SHR and NTR suggesting that (i) the dose of indomethacin used in this study was sufficient to at least partially block prostaglandin formation (further demonstrated by the significant inhibition of the cardiovascular effect of arachidonic acid) and (ii) prostaglandins play a role in modulating renin release under normal circumstances as suggested by others (see Introduction). It is possible that higher doses of indomethacin could have had more profound inhibitory effects on PRA changes caused by captopril. This is unlikely in SHR since propranolol caused a virtual abolition of the PRA response, but this cannot be ruled out in NTR. Higher doses of indomethacin were found to be toxic.

Summary. Captopril reduced blood pressure and increased PRA in both SHR and NTR. Both propranolol and indomethacin caused significant reductions in resting PRA of both SHR and NTR but only propranolol caused an inhibition of the PRA changes caused by captopril. Furthermore, since propranolol was highly effective only in SHR and captopril decreased blood pressure much more in SHR than NTR, the increase in PRA caused by captopril in SHR was probably reflexly mediated by compensatory sympathetic activation, all of which actions were a consequence of reducing angiotensin II formation.

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Cl^- Diffusion Potential of Nutrient Membrane of Frog Gastric Mucosa (40698)

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Forte *et al.* (1) were the first to show that isethionate ion like SO_4^{2-} replacing Cl^- in the bathing media of the *in vitro* frog gastric mucosa reversed the transmembrane potential difference (PD) across the mucosa. In another aspect of their investigation, changing from 15 to 60 m*M* Cl^- in the nutrient solution with isethionate as the replacement ion for Cl^-, they obtained a Cl^- diffusion potential of 54 mV per 10-fold change in Cl^- concentration. The latter approached the value of 58 mV predicted by the Nernst equation. In a model involving conductance pathways on the nutrient membrane (2), their results would suggest that the Cl^- conductance pathway was the only high conductance pathway across the nutrient membrane. On the other hand, Spangler and Rehm (2) obtained a diffusion potential of 19 mV using SO_4^{2-} as the replacement ion for Cl^-. Their results suggest that Cl^- contributes about one-third to the total conductance of the nutrient membrane. In the work of Spangler and Rehm (2), the relative conductance of an ion was assessed by determining the effect on the PD resulting from rapid changes of the ionic composition of the nutrient fluid. According to their considerations, if readings of the PD were taken during the first few minutes, there would be no marked changes in the characteristics of the mucosa arising from ionic changes. If, then, this method were applied to isethionate as well as SO_4^{2-} as the replacement ion for Cl^-, would the Cl^- diffusion potential give essentially the same results for both replacement ions or might differences still arise due to differences in permeability between isethionate and SO_4^{2-}? The results of this investigation are described herein.

Methods. The experiments were performed on gastric mucosae of *Rana pipiens* with an *in vitro* method described in detail elsewhere (3). Two pairs of electrodes were used, one for sending current across the mucosa and the other for measuring the PD. The resistance was obtained as the change in PD per unit of applied current. The H^+ secretory rate was determined by the pH stat method introduced by Durbin and Heinz (4). The pH of the secretory side was maintained at 4.90. The regular nutrient bathing solution contained (in m*M*): Na^+, 102; K^+, 4; Ca^{2+}, 1; Mg^{2+}, 0.8; Cl^-, 81; HCO_3^-, 25; phosphate, 1.0; and glucose, 10; and the secretory bathing solution contained: Na^+, 102; K^+, 4; and Cl^-, 106. Both sides of the mucosa were gassed with 95% O_2 and 5% CO_2. Histamine was added to the nutrient solution to a concentration of 10^{-4} *M*. Isethionate or SO_4^{2-} replaced Cl^-. For experiments with divalent ions, the sucrose concentration was altered to insure isosmolality. Generally the nutrient solution was drained and washed once with the new solution. The first PD reading could be taken about 15 sec after the change. Further details are provided elsewhere (2).

The PD of the nutrient side is considered relative to the secretory side.

Results. Figure 1 represents the results of a typical experiment in which the nutrient solution containing 6 m*M* Cl^- was replaced with one containing 81 m*M* Cl^-. In the first part of the experiment, SO_4^{2-} replaced Cl^- in the nutrient solution and, in the second part, isethionate replaced Cl^-. The change to 81 m*M* Cl^- caused the PD to attain a maximum value within 2–3 min. The maxima were, respectively, about 26 and 22 mV above the control value. During this period, the resistance showed a small decrease. The H^+ secretory rate increased to a small extent during the first part and showed no change in the second part. Generally, the H^+ secretory rate showed a small but significant increase. Reversal of ion replacement (that is, isethionate first and SO_4^{2-} second) gave essentially the same results.

Table I summarizes the results of three kinds of concentration changes in the nutrient solution: (i) from 6 m*M* Cl^- to 81 m*M* Cl^-, (ii) from 81 m*M* Cl^- to 6 m*M* Cl^-, and (iii)

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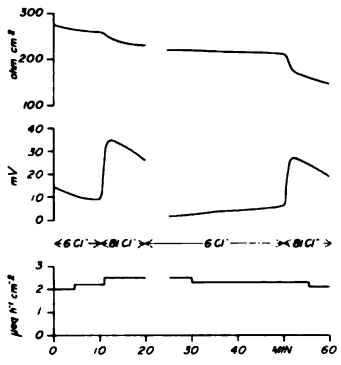

. 1. Effect of changing 6 m*M* Cl^- in nutrient n to 81 m*M* Cl^- on resistance, PD, and H^+ secrete versus time. In the first part (0 to 10 min) SO_4^{2-} d Cl^-. The break in the curve represents several gs of the nutrient membrane with 6 m*M* Cl^- and nate ion in place of Cl^-.

15 m*M* Cl^- to 60 m*M* Cl^- for both onate and SO_4^{2-} in place of Cl^-. The s were averaged irrespective of the order l replacement. We note further that, for ges from low to high concentrations of the maximum change in PD was re- d as well as the corresponding changes sistance and H^+ secretory rate. For ges from high to low concentrations of the data at inflection points were re- d as explained in the discussion.

first note that the change in PD never ded 20 mV per 10-fold change in Cl^- concentration for either SO_4^{2-} or isethionate ion. The resistance either did not change or showed a small significant change. The H^+ secretory rates showed similar results. In experiments of Spangler and Rehm in which Cl^- was changed in the nutrient solution from 81 m*M* Cl^- to 4 m*M* Cl^- using SO_4^{2-} as the replacement ion, they obtained a decrease in PD of 18.7 mV per 10-fold change and no significant change in resistance. For similar changes in concentration, our results gave similar changes in PD and resistance. From Table I, line 2, we determined that, for a 10-fold change, the PD decreased by 19.3 mV compared to 18.7 mV and that the change in resistance was not significant. Their data suggest that a decrease in H^+ secretory rate is expected, as indicated in our Table I. For reverse changes in concentration, they obtained an increase in PD of 16.2 mV compared to our increase of 17.9 mV per 10-fold change. Both groups found that $|\Delta PD|$ for changes from low to high concentrations of Cl^- was less than $|\Delta PD|$ for the reverse changes. Moreover, isethionate gave values of $|\Delta PD|$ somewhat less than those obtained with SO_4^{2-}.

Lastly, in changes in Cl^- concentration from 15 to 60 mV, we obtained, according to the data of Table I, $\Delta PD = 20$ mV for SO_4^{2-} replacing Cl^- and $\Delta PD = 18$ mV for isethionate replacing Cl^- per 10-fold change in con-

LE I. Effects of the Change in Cl^- Concentration in the Nutrient Solution on Resistance, PD, and H^+ Secretory Rate of the Frog Gastric Mucosa[a]

) Final (m*M*)	No. of Expts	*R* (Ω-cm²)	Δ*R* (Ω-cm²)	PD (mV)	ΔPD (mV)	$\dot{H}$ (μeq hr⁻¹cm⁻²)	$\Delta\dot{H}$ (μeq hr⁻¹cm⁻²)
			Cl^- substituted with SO_4^{2-}				
81	17	197 ± 55	−30 ± 23 ($P < 0.01$)	−5.1 ± 8.4	20.2 ± 5.3 ($P < 0.01$)	2.73 ± 0.80	0.48 ± 0.53 ($P < 0.01$)
6	12	191 ± 67	11 ± 26 ($P > 0.10$)	24.5 ± 6.5	−21.8 ± 3.7 ($P < 0.01$)	4.68 ± 1.73	−0.52 ± 0.52 ($P < 0.01$)
60	7	139 ± 49	−17 ± 15 ($P < 0.05$)	−1.4 ± 5.9	12.1 ± 1.9 ($P < 0.01$)	4.72 ± 1.52	0 ± 0 ($P = 1.0$)
			Cl^- substituted with isethionate ion				
81	17	187 ± 59	−24 ± 24 ($P < 0.01$)	−6.1 ± 9.1	19.7 ± 4.7 ($P < 0.01$)	2.83 ± 0.81	0.38 ± 0.48 ($P < 0.01$)
6	10	224 ± 59	8 ± 13 ($P > 0.05$)	24.9 ± 5.8	−18.7 ± 2.5 ($P < 0.01$)	3.84 ± 0.89	−0.23 ± 0.29 ($P < 0.05$)
60	7	148 ± 70	−22 ± 19 ($P < 0.05$)	−0.2 ± 6.8	10.9 ± 1.1 ($P < 0.01$)	4.56 ± 0.94	0.12 ± 0.24 ($P > 0.80$)

lues are means ± SD. Columns labeled *R*, PD, and $\dot{H}$ refer, respectively, to the control values of resistance, embrane potential difference, and H^+ secretory rate. Columns labeled Δ*R*, ΔPD, and $\Delta\dot{H}$ refer to changes in ee parameters following the change to the final solution, values corresponding to the maximum $|\Delta PD|$ or the an inflection point. *P* values were calculated using the paired-variates method of the Student's *t* test.

centration. These changes differ markedly from the 54 mV change reported by Forte *et al.*

Discussion. To explain the difference in values pertaining to the Cl^- diffusion potential, we refer to the work of Spangler and Rehm (2). They found that the time course for a rapid change in concentration was essentially that predicted on the basis of a model comprising a membrane in series with a diffusion barrier. From their analyses, it is evident that we are mainly concerned with the changes that occur in the first few minutes since it appears that the characteristics of the mucosal cells do not alter substantially during this period. For a change from a low to a high concentration of a permeant ion, they showed that $|\Delta PD|$ versus time passed through a maximum. For a change from a high to a low concentration, $|\Delta PD|$ versus time gave an inflection point, although, in actual practice, the PD sometimes leveled off for several minutes before again changing. In Table I, we used these criteria recording data at maxima and inflection points.

In terms of this model, we shall now attempt to explain the differences between Spangler and Rehm using SO_4^{2-} as the replacement ion for Cl^- and Forte *et al.* using isethionate. From our data, it is evident that any permeability differences between SO_4^{2-} and isethionate give only minor differences in the values of ΔPD per 10-fold change in Cl^- concentration. Therefore, while Spangler and Rehm observed the changes in PD shortly after the substitution, it would appear that Forte *et al.* observed the changes in PD, although not specifically stated in their paper, at or near the new steady state of th concentrated Cl^- solution. Thus, the change in H^+ secretory rate which th served does not assure that the increas concentration will not eventually aff electrogenic Cl^- pump (3) located in cretory membrane. Hence, if the m ments are made later than the first fe utes, the effect obtained may be larg to the Cl^- pump itself or other chan duced by the new Cl^- concentration.

Our data in conformity with those o gler and Rehm indicate that the Cl ductance pathway contributes abou third to the total conductance of the n membrane in regular Cl^- nutrient so bathing the frog gastric mucosa.

Summary. Replacement of Cl^- with gives a Cl^- diffusion potential of ab mV across the nutrient membrane per change in Cl^- concentration, suggesti the basis of the Nernst equation, that t conductance pathway contributes abo third to the total conductance of this brane in regular Cl^- solutions bathi frog gastric mucosa. Isethionate ion change in PD of about 17 mV per change which is quite close to the val tained with SO_4^{2-}.

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Bombesin Stimulates Gastrin Secretion in the Rat without Increasing Serum Calcitonin (40699)[1, 2]

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Gastrin and its synthetic analog, pentagastrin, are potent calcitonin (CT) secretagogues in several mammalian species, including man (1–5). However, in the laboratory rat we have not found either gastrin or pentagastrin to be a potent CT secretagogue (6–8). This conclusion was based both on *in vivo* studies where large doses of gastrin or pentagastrin were given i.v. (6) and on *in vitro* studies where thyroid glands were incubated in medium containing various concentrations of gastrin or pentagastrin (7). Similar independent *in vivo* studies in the rat by Garel and associates (9, 10) have led them to this conclusion, also.

The present study was designed to see whether or not endogenous secretion of gastrin in the rat would act as a CT secretagogue. Since the structure of rat gastrin has not been elucidated, it was possible that some unique unrecognized feature of homologous rat gastrin might endow it with CT secretory activity in the rat. To provoke gastrin release, we employed the amphibian tetradecapeptide, bombesin, which has been shown to be a potent and highly efficacious secretagogue in the dog, cat, and human (11–14). Our results show that bombesin stimulates gastrin release in the rat as well but that the increased gastrin does not, in turn, provoke demonstrable increases in blood levels of CT.

Materials and methods. Animals. Male Sprague–Dawley rats, 5–8 weeks old and weighing 120–200 g, were obtained from ARS/Sprague–Dawley (Madison, Wis.). Following their receipt, the rats were maintained on Wayne Laboratory Blox (Granville Milling Co., Creedmore, N.C.) until use. The rats were fasted for 18–26 hr just before use.

Procedures. Separate groups of rats were lightly anesthetized with ether and injected i.v. (tail vein) with synthetic bombesin (Bachem, Inc., Torrance, Calif.). In one experiment synthetic cyclic ovine somatostatin (SRIF, Beckman Co., Palo Alto, Calif.) also was injected i.v., and it was given 1 min before bombesin. Both peptides were dissolved in 0.15 *M* NaCl to give the desired dose in 0.5 ml, and this volume was injected. Control rats received 0.5 ml of 0.15 *M* NaCl. At the desired time intervals after injection, blood samples were drawn under ether anesthesia by cardiac puncture using a 20-gauge, 1-in. needle. Samples were placed on ice and allowed to clot. Serum was obtained by centrifugation within 1 hr of blood collection. Each serum sample was subdivided, one portion being analyzed freshly for calcium and inorganic phosphorus and the other being stored frozen at −20° until subjected to radioimmunoassays.

Analytical procedures. Calcium and inorganic phosphorus in serum were determined using an AutoAnalyzer II (Technicon, Tarrytown, N.Y.). The procedures are modifications of previously described analytical procedures (15–17). CT in serum was measured using a radioimmunoassay for rat CT which has been described in detail previously (7, 8). The homologous assay utilizes a chicken antiserum to rat CT (final dilution 1:10,000) and purified native rat CT for iodination and as unlabeled reference standard. Gastrin in serum was measured using a radioimmunoassay described earlier in detail (18, 19). A guinea pig antiserum to porcine gastrin (final dilution 1:100,000) was used, and synthetic human G-17 (ICI, England) was employed for iodination and as unlabeled reference standard.

Statistical analyses. For serum calcium and phosphorus, the data were subjected to anal-

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[2] Portions of this work were presented at the 63rd Annual Meeting of the Federation of American Societies for Experimental Biology, Dallas, Tex., April 5, 1979 (Fed. Proc. 38, 850 (1979)).

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ysis of variance, and standard errors (SEM) were calculated from the residual error term of the appropriate analysis (20). The significance of differences between mean values was evaluated using either the *F* test or the multiple comparisons test of Schéffe (20). Radioimmunoassay values were subjected to nonlinear regression analysis (21) on an IBM 1130 computer. For experiments where assay values in most groups exceeded the lower limit of assay detectability, mean values ± SEM were calculated; where most values were below limits of detectability, individual values are presented. For hormone values, the significance of differences between groups was determined using the nonparametric rank sum test of Wilcoxon for unpaired measurements (20).

Results. Results shown in Fig. 1 are representative of those obtained in several identical experiments conducted. Figure 1A shows that bombesin, given in doses of 10 or 50 μg i.v. for 5 or 30 min, produced no change in serum calcium compared to control rats given saline i.v. for 5 min. In this experiment no change in serum phosphorus was observed either, although a small but significant hypophosphatemia occurred after bombesin in some of the experiments not shown. Figure 1B shows the values for serum CT and gastrin in the same rats. Both doses of bombesin produced a significant increase of approximately twofold in gastrin within 5 min, and by 30 min even greater increases were apparent. In spite of the marked hypergastrinemia induced by bombesin, serum CT remained undetectable in all four groups given bombesin, and this was confirmed in other experiments not shown.

To ensure that a possible bombesin-induced increase in serum CT was not inadvertently missed, both dose–response and time-course studies were conducted using bombesin (Table I and Fig. 2). Figure 2A and Table I show that no substantial effects of bombesin on serum calcium or phosphorus were observed. The results in Fig. 2B show that large and significant increases in serum gastrin occurred between 5 min and 2 hr after bombesin at a dose of 50 μg/rat. Results shown in Table I illustrate that the doses of bombesin used in most of the studies (10 and 50 μg/rat) were appropriate, since a dose higher than 50 μg/rat did not lead to a further increase in serum gastrin 30 min after injection. Both Fig. 2B and Table I illustrate that,

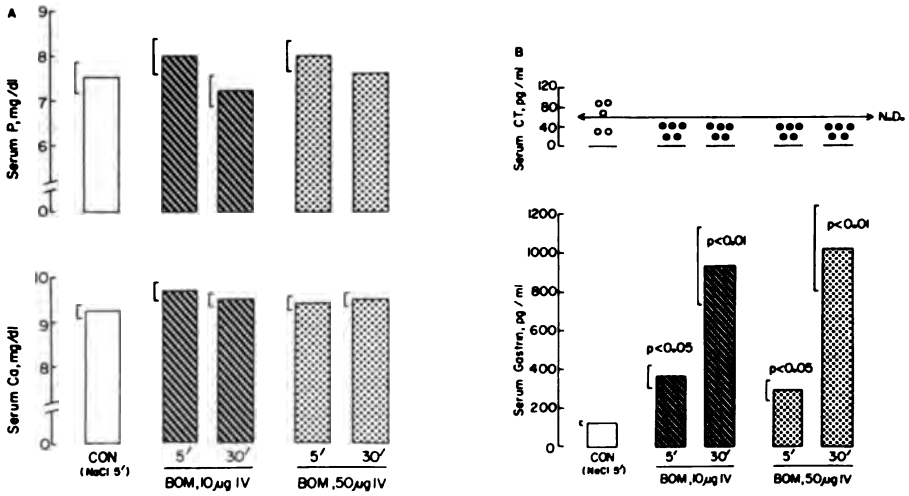

FIG. 1. (A) Concentrations of calcium and inorganic phosphorus in serum of 7-week-old rats injected with bombesin (BOM) either 5 min (5') or 30 min (30') earlier. The height of each bar represents the mean value for a separate group of five rats, and the bracket shows the SEM. The control rats (CON) received saline i.v. 5 min before blood collection. All rats had been fasted for 21 hr. (B) Concentrations of gastrin and CT in the same rats shown in (A). Bars and brackets in lower graph represent mean values ± SEM. Upper graph shows individual values for each rat; horizontal arrow represents lower limit of detectability for the immunoassay (N.D. = not detectable). *P* values refer to comparison of mean values of rats given BOM to CON rats.

TABLE I. SERUM CALCIUM, GASTRIN, AND CT 30 MIN AFTER INJECTION OF VARIOUS DOSES OF BOMBESIN[a]

Dose of bombesin (μg)	No. rats	Serum Concentrations: Calcium (mg/100 ml)	Gastrin (pg/ml)	CT (pg/ml)
) (Control)	6	9.53 ± 0.12	50 ± 10	ND[b]
).016	5	9.41 ± 0.12	30 ± 13	ND
).08	6	9.55 ± 0.12	40 ± 11	ND
).4	5	9.36 ± 0.12	38 ± 12	ND
!.0	6	9.20 ± 0.12	100 ± 43	ND
).0	6	9.35 ± 0.12	152 ± 59*	ND
).0	6	9.48 ± 0.12	269 ± 82**	ND
).0	6	9.53 ± 0.12	204 ± 58*	ND

[a] Each value presented represents the mean ±SEM a separate group of rats. Differs from control group: ' < 0.05, ** $P < 0.01$. Control rats received 0.5 ml 0.15 NaCl i.v.
[b] Not detectable (all values < 30 pg/ml).

spite the increases in serum gastrin proced by bombesin, the hypergastrinemia is not accompanied by demonstrable eletions in serum CT. In fact, serum CT reained low or undetectable in both control d bombesin-treated rats. These low or untectable levels are characteristic for fasted ts of this age (6).

In one experiment (Fig. 3) bombesin and matostatin (SRIF) were given both alone d together. No effects on serum calcium or phosphorus were observed (Fig. 3A). Figure 3B shows that bombesin alone produced a marked increase in serum gastrin. SRIF alone did not alter serum gastrin levels, but when SRIF was given with bombesin it significantly reduced, but did not eliminate, the bombesin-induced hypergastrinemia. In this particular experiment almost all of the rats had detectable levels of serum CT (Fig. 3B, top graph), the values ranging from ≈ 100 to 200 pg/ml. However, no significant changes in serum CT were observed in any of the experimental groups receiving SRIF or bombesin.

Discussion. In several mammals, including man, the gastrointestinal hormones, gastrin and cholecystokinin, clearly are CT secretagogues (1–5). In the pig, we even have shown that gastrin is an effective stimulator of CT release at blood levels of gastrin which occur normally after a meal (8). Surprisingly, however, in the rat gastrin is weak or ineffective in provoking CT release as judged by both *in vivo* or *in vitro* experiments where thyroid glands were exposed to high doses of pentagastrin and human or porcine heptadecapeptide gastrin (6–8). Since the biological activity of gastrin resides in the C-terminal tetrapeptide amide, a region highly conserved in the mammalian gastrins which have been se-

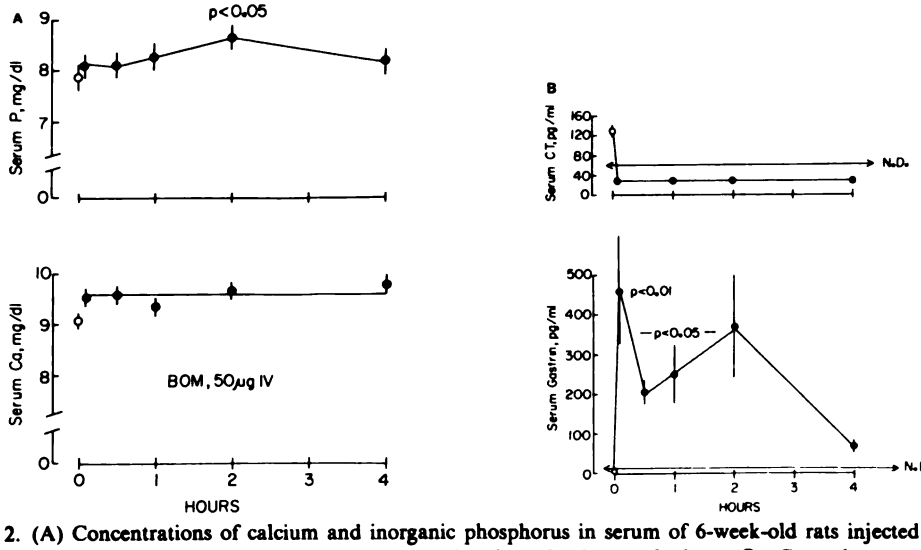

FIG. 2. (A) Concentrations of calcium and inorganic phosphorus in serum of 6-week-old rats injected with a gle dose of bombesin (BOM) and bled at times ranging from 5 min to 4 hr later (●). Control rats not given nbesin (○) received an injection of saline and were bled 5 min later. Each point represents the mean value for a arate group of five rats, and vertical lines shown the SEM. (B) Concentrations of gastrin and CT in the same rats wn in (A). See Fig. 1B legend for additional details.

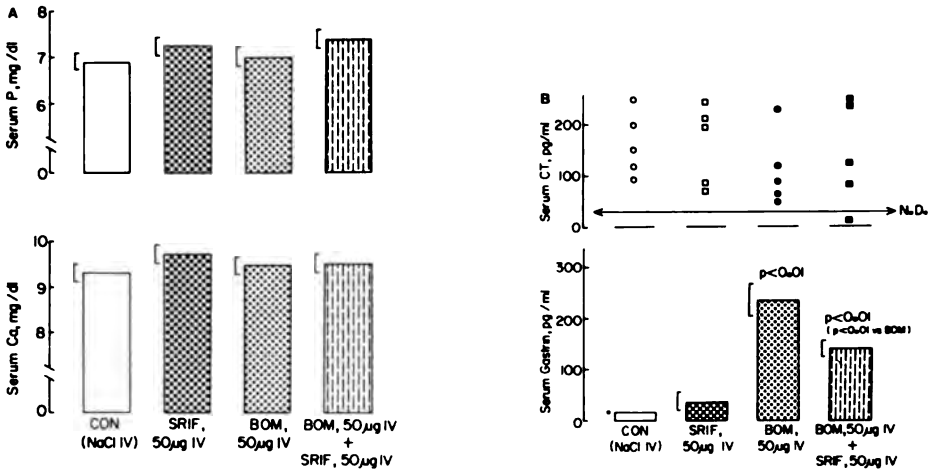

FIG. 3. (A) Concentrations of calcium and inorganic phosphorus in serum of 7-week-old rats injected with somatostatin (SRIF) or bombesin (BOM) alone or in combination. Rats were bled 30 min after injection; control rats (CON) received saline i.v. 30 min before blood collection. All rats had been fasted for 20 hr. See Fig. 1A legend for additional details. (B) Concentrations of gastrin and CT in the same rats shown in (A). See Fig. 1B legend for additional details.

quenced (22), it seemed unlikely that failure to elicit a CT response in the rat could be attributed to use of heterologous gastrins. However, since the structures of gastrin moieties in the rat, including G-17, are not known, it was possible that some unappreciated special feature of the rat gastrin molecule is required for CT-secretory activity in this species. Therefore, in the present study we attempted to provoke secretion of endogenous rat gastrin in large amounts to see whether or not the hypergastrinemia would lead to increased secretion of CT. For a gastrin secretagogue we chose bombesin, an amphibian tetradecapeptide which has been shown to be effective in a number of mammalian species (11–14).

Our findings clearly show that bombesin is effective in promoting gastrin secretion in the rat (Figs. 1B, 2B, and 3B and Table I) as has been shown with other mammals. The effect on gastrin in the rat is dose dependent (Table I), rapid in onset (Figs. 1B and 2B), and lasts for several hours after a single i.v. injection (Fig. 2B). Furthermore, the increase in gastrin after bombesin can be partially inhibited by somatostatin (Fig. 3B), which itself did not influence basal serum gastrin in these experiments. It is of interest (i) that somatostatin did not inhibit basal gastrin in the rat, as it does in a variety of other mammalian species, including the pig (19), and (ii) that unlike the case in man (23) and the pig (24), somatostatin did not inhibit CT secretion in the rat (Fig. 3B).

Despite the fact that bombesin produced a marked hypergastrinemia in the rat, in none of the experiments did we find an increase in serum CT. This was true both for experiments where basal serum CT was close to or below the lower limit of assay detectability (Fig. 1B and Table I) and those where it was clearly measurable (Figs. 2B and 3B). In none of the experiments did we observe any consistent effects of bombesin on serum calcium or phosphorus. Our findings in the rat are in marked contrast to those in the pig where bombesin-induced hypergastrinemia rapidly is followed by six- to eightfold increases in CT secretion (25). The failure to observed CT secretion in the rat during hypergastrinemia confirms our previous impression that gastrin is a poor CT secretagogue in this species. We conclude that some other unidentified gastrointestinal factor or event must be involved in the CT response observed in the rat following feeding or administration of calcium-free solutions (8, 10, 26, 27).

Summary. Bombesin was administered to young fasted rats, and blood levels of immunoreactive gastrin and calcitonin (CT) were measured. Doses of bombesin up to 100 μg/

..v. did not affect serum calcium or phosrus. Five minutes after bombesin, 10 or g/rat, serum gastrin increased 1- to 2-fold , by 30 min, it was elevated 5- to 10-fold. ıe-course and dose–response studies ved that doses of 10–100 μg/rat increased rin and that gastrin rose within 5 min r 50 μg/rat and remained elevated for at t 2 hr. Despite bombesin-induced hyperrinemia, CT remained low or undetecta- (<50–100 pg/ml) at all times. In one ly, somatostatin (50 μg/rat i.v.), given toıer with bombesin, inhibited the rise in ım gastrin by 50%, but serum CT, which detectable, was unaffected by either bomn or somatostatin. We conclude that bomn stimulates gastrin release in the rat, but elevated gastrin does not promote CT etion. The results support our earlier find- which indicate that gastrin is weak or fective in the rat as a CT secretagogue.

he technical assistance of Ms. Deloris B. Alston and Lillie T. Williams is gratefully acknowledged.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 442–444 (1979)

Phenylbutazone Plasma Binding: Effects of Salicylic Acid, Indomethacin, and Dicloxacillin (40700)

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Virtually all drugs and substances that are highly bound to human serum albumin are potentially capable of displacing each other from their binding sites in the protein molecule. This type of drug interaction can influence significantly the therapeutic and toxic effects of the competing drugs. Phenylbutazone, a nonsteroidal anti-inflammatory drug, has been shown to displace anticoagulants, antidiabetics, and antibiotics from their binding sites in human serum albumin resulting in enhanced therapeutic or toxic effects of these displaced drugs (1–4). Conversely, albumin-bound phenylbutazone can also be displaced by other drugs and substances such as anticoagulants, antidiabetics, and fatty acids (5, 6). Despite the enormity of possible drug interactions involving protein binding, however, relatively few studies have been done to investigate drug binding competition. In this report, we examine the effects of other highly bound drugs (salicylic acid, indomethacin, and dicloxacillin) on the binding of phenylbutazone to human plasma proteins and discuss the clinical significance of these drug binding interactions.

Materials and methods. Phenylbutazone, salicylic acid, and indomethacin were obtained from Sigma Chemical Company, St. Louis, Missouri, while sodium dicloxacillin was obtained from Bristol Myers Company, Syracuse, NY. Whole human plasma was provided by the blood bank of Ramathibodi Hospital, Bangkok, Thailand.

The binding of phenylbutazone to human plasma proteins was investigated using the equilibrium dialysis technique as described by McMenamy (7) with some modifications as follow: Cellulose dialysis tubing (Fischer Co., Pittsburg, Pa.) was soaked in distilled water for 3 days with water changes twice daily. After the washing, inflated dialysis bags with glass spacers inside were prepared and dried. Protein solution (human plasma) was pipetted into the dialysis bags and the bags were inserted into test tubes containing 0.1 *M* phosphate buffer, pH 7.4, with various concentrations of phenylbutazone. The test tubes were stoppered and attached to a multipurpose rotator (Model 15 V, Scientific Industries, Inc., Springfield, Mass.) with an inclination of about 30°. Dialysis was done at 4°C for 24 hr. Preliminary experiments showed that equilibration was complete within 24 hr and that there was no significant binding to the dialysis bag or to the glass. Phenylbutazone concentrations in the inside and outside solutions were determined using the spectrophotometric method described in detail by Burns *et al.* (8). The extent of binding was calculated from the following formula:

$$\% \text{ Binding} = \frac{C_t - C_f}{C_t} \times 100, \qquad [1]$$

where C_t = total phenylbutazone concentration (inside the bag), and C_f = concentration of free phenylbutazone (outside the bag).

The results obtained by the technique of putting the drug in the inside solution were essentially the same as that of putting the drug in the outside solution. The latter technique, however, was chosen in our experiments to avoid dilution of the protein solution and because it was easier to perform.

Competition with phenylbutazone plasma binding was studied by adding a constant concentration of the competitor drug to the outside solution of the dialysis tubes. Salicylic acid and indomethacin were dissolved in ethanol while sodium dicloxacillin was dissolved in distilled water. Preliminary experiments showed that ethanol, in the strengths used (3.5–7.0%), did not interfere with phenylbutazone plasma binding.

The effect of salicylic acid on the binding of phenylbutazone to 3.5% human serum albumin (Sigma Chemical Co.) was also deter-

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mined by equilibrium dialysis and the binding data were analyzed using the Scatchard plot (9).

Results. Displacement of phenylbutazone from its binding sites in human plasma was observed with salicylic acid and indomethacin while there was no apparent change with dicloxacillin (Fig. 1). In a wide range of total plasma phenylbutazone concentrations, the percentage binding of phenylbutazone was decreased when either salicylic acid or indomethacin was added. At a total salicylic acid concentration of about 200 μg/ml (which is within the range of therapeutic concentrations for anti-inflammatory effects), the percentage plasma binding of phenylbutazone was decreased by about 4–14% resulting in a two- to fourfold increase in the concentration of the free drug. With indomethacin concentration of about 200 μg/ml, which is extremely beyond the range of therapeutic concentrations obtained clinically (10), the decrease in phenylbutazone plasma binding was about 2–4%. Dicloxacillin, even at a very high concentration of about 250 μg/ml, did not significantly alter the extent of phenylbutazone plasma binding.

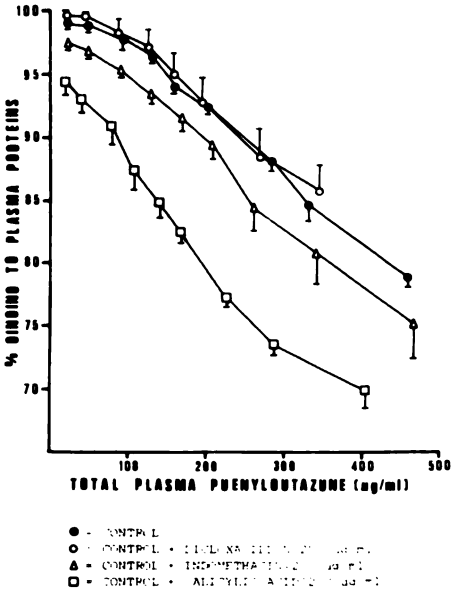

FIG. 1. Plasma protein binding of phenylbutazone in the presence of dicloxacillin, indomethacin, and salicylic acid. The binding of phenylbutazone to plasma proteins in the presence of salicylic acid (200 μg/ml), indomethacin (200 μg/ml), and dicloxacillin (250 μg/ml) was determined by equilibrium dialysis as described under Materials and Methods. The percentage of bound phenylbutazone was plotted against the total phenylbutazone concentration in the plasma. Each point represents the mean ± SE of three separate experiments.

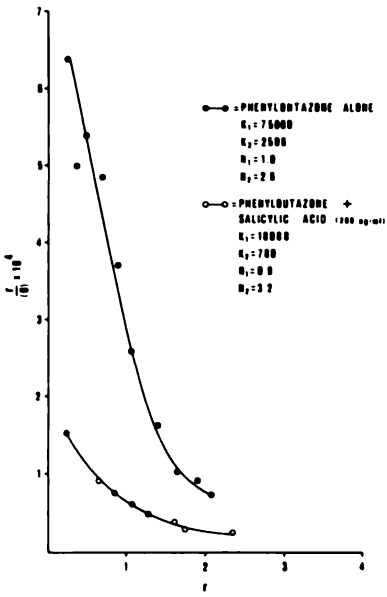

FIG. 2. Effect of salicylic acid on the binding of phenylbutazone to 3.5% human serum albumin. The binding data were obtained by equilibrium dialysis and analyzed according to the Scatchard plot: r = moles phenylbutazone per mole of albumin, (D) = concentration of free phenylbutazone, K = affinity constant, N = number of binding sites. Subscripts 1 and 2 refer to the high-affinity and low-affinity types of binding sites, respectively.

The effect of salicylic acid on the binding of phenylbutazone to 3.5% human serum albumin is shown in Fig. 2. The affinity constants (K_1 and K_2) of phenylbutazone as estimated by the Scatchard plot were dramatically reduced in the presence of salicylic acid (200 μg/ml) while there was no apparent change in the number of binding sites (n_1 and n_2).

Discussion. The displacement of protein-bound phenylbutazone caused by salicylic acid has important clinical implications because the concentration (200 μg/ml) used in this experiment could be easily reached with usual doses of salicylates in the treatment of rheumatoid arthritis and related diseases to achieve antiinflammatory effects. This interaction may enhance the toxic effects of phenylbutazone and salicylates when these drugs are combined in the therapy of arthritic dis-

eases. Although the decrease in the percentage binding is only 4–14%, the resulting increase in the concentration of the free drug is greatly magnified (two- to fourfold). Even if phenylbutazone is given at one-half the usual dose, when combined with salicylates, toxic levels of the free drug could still be reached while there may be actually a decrease in the total plasma concentration.

On the other hand, the slight displacement caused by indomethacin is not likely to be of clinical importance because this displacement was observed at an extremely high concentration of indomethacin not encountered clinically. Surprisingly, dicloxacillin did not appear to affect the plasma binding of phenylbutazone. This indicates that highly bound drugs do not necessarily displace each other from their binding sites in the protein molecule. A possible explanation is that they are bound at different mutually noninteracting sites in the albumin molecule.

The binding competition between salicylic acid and phenylbutazone is further elucidated by experiments using human albumin. Both the high affinity constant (K_1) and the low affinity constant (K_2) were decreased while the number of binding sites (n_1 and n_2) remained practically unchanged (Fig. 2). This indicates that the two drugs compete for the same binding sites in the albumin molecule.

Summary. The effects of three highly bound acids, viz salicylic acid, indomethacin, and dicloxacillin, on the binding of phenylbutazone to human plasma proteins were studied by equilibrium dialysis. Salicylic acid was found to displace phenylbutazone from its binding sites to a degree which might be clinically significant. Indomethacin caused a slight decrease in the percentage binding of phenylbutazone which is not likely to be of clinical importance. Dicloxacillin, however, did not appear to affect the plasma protein binding of phenylbutazone suggesting that the two drugs could be bound at mutually noninteracting sites in the protein molecule.

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nhibition of Mineralization by Experimental Lathyrism during Matrix-Induced Endochondral Bone Differentiation (40701)

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various types of collagens have re- been implicated in cell attachment, cell ntiation, and morphogenesis (1). There ɔwing realization of the importance of inked collagenous matrix for mineral- of bone (2–4). The availability of spe- ;ents that inhibit collagen cross-linking ; to experimental lathyrism (5) enables erimental scrutiny of the influence of ɔndition on bone differentiation and ılization. The use of the matrix-in- endochondral bone differentiation sys- aracterized in our laboratory (6–8) per- .e study of discrete stages of endochon- ɔne differentiation, such as mesenchy- ll proliferation, chondrogenesis, carti- alcification, osteogenesis, and bone ılization. This system avoids many of fficulties inherent in the use of the seal growth plate. The present study ɔes the influence of β-aminopropioni- APN)-induced experimental lathyrism differentiation of endochondral bone.

erials and methods. Animals. Thirty-six 25-day-old (50–60 g) male rats of Evans strain were divided into two . The control group was pair fed with dered rat chow while the experimental was fed the same diet supplemented .3% (w/w) of β-aminopropionitrile fu- : (BAPN; Aldrich Chemical Co.). At d of 2 weeks the rats were implanted aneously with demineralized diaphy- ɔne matrix powder in the thoracic re- s described earlier (6). The rats in each were pair fed the designated diet until mination of the experiment. The day lantation was designated as Day 0 and ts were utilized for different experi- on designated days as described in the

The present investigation was per- l during the following distinct stages of -induced endochondral bone differen- : (i) proliferation of mesenchymal cells on Day 3, monitored by the activity of ornithine decarboxylase (ODC); (ii) chondrogenesis on Day 7 as evidenced by $^{35}SO_4$ incorporation into proteoglycans; (iii) cartilage calcification on Day 9 as indicated by alkaline phosphatase and ^{45}Ca incorporation; and (iv) osteogenesis and bone mineralization on Days 14 and 21 as quantitated by alkaline phosphatase activity and ^{45}Ca incorporation. The proximal tibial epiphyses and tibial metaphyses were utilized as internal controls for assessing the influence of BAPN on chondrogenesis and osteogenesis, respectively.

Ornithine decarboxylase (ODC) assay. Matrix-induced plaques (implants) were dissected out on Day 3, cleaned of adherent tissue, and homogenized in ice-cold 50 m*M* Tris–HCl buffer containing 5 m*M* dithiothreitol and 1 m*M* EDTA at pH 7.4. The samples were centrifuged at 30,000 *g* in a Beckman JA-21 centrifuge for 30 min and the supernatant fractions were used for the ODC assay. The assay was performed as described (9) and the results expressed as pmoles of $^{14}CO_2$ released from L-[1-^{14}C]ornithine per hour per milligram protein. Protein was determined as described (8, 9) using bovine serum albumin as standard.

$^{35}SO_4$ incorporation. Sodium (^{35}S) sulfate in saline was injected intravenously at a dose of 1 μCi/g body wt. The rats were killed 2 hr after injection; the plaques were dissected out and cleaned, and a portion of each was weighed and homogenized in ice-cold 10% (w/v) trichloroacetic acid (TCA) containing 10 m*M* nonradioactive sodium sulfate. The samples were kept cold for 30 min and centrifuged at 20,000 *g* for 15 min. Aliquots of the acid-soluble supernatant fraction were used for determination of ^{35}S radioactivity. The acid-insoluble precipitate was washed twice with 5 ml of ice-cold 10% TCA and dissolved in 0.5 ml 23 *M* formic acid at 90° for 10 min and transferred to scintillation

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vials, and the radioactivity was determined in a liquid scintillation counter. The results were expressed as cpm/mg of tissue and statistically evaluated by means of the Student's *t* test. A portion of each plaque was also used for determination of alkaline phosphatase activity as described earlier (6).

^{45}Ca incorporation and alkaline phosphatase activity. $^{45}CaCl_2$ was injected intravenously via the caudal vein in saline at a dose of 1 μCi/g body wt 2 hr prior to the termination of the experiment. The plaques were dissected out and homogenized in ice-cold 0.15 *M* NaCl containing 3 m*M* $NaHCO_3$ and centrifuged at 20,000 *g* for 15 min at 4°. Alkaline phosphatase activity in the supernatant was determined as described previously (6). The saline-insoluble sediment representing the mineral fraction was suspended in 10 ml 0.1 *M* $CaCl_2$ and stirred for 30 min. After centrifugation at 3000 *g* the supernatant fractions were discarded. The sediment fractions were washed twice in 10 ml of deionized glass distilled water and the pellets were stirred for 2 hr in 10 ml 0.5 *M* HCl and centrifuged at 3000 *g* for 5 min. Radioactivity in the aliquots of the supernatant acid extract were determined in a Beckman liquid scintillation counter. The ^{45}Ca incorporation was expressed as cpm/mg tissue and represented the rate of mineralization during the 2-hr pulse of $^{45}CaCl_2$. Alkaline phosphatase activity was expressed as units/mg protein. The statistical significance was evaluated by means of the Student's *t* test.

Calcium determination. Aliquots of the acid-soluble extracts of the sediment were diluted in 0.5% HCl containing 0.1% lanthanum oxide for determination of calcium content by atomic absorption spectrophotometry. The results were expressed as μg calcium/mg tissue and represented the total extent of mineralization throughout the experimental duration.

Preparation and assay of bone and tooth matrices from lathyritic rats. Thirty-six, 21-day-old (45–50 g), Long–Evans rats were divided into two groups. The control group was fed Purina rat chow while the lathyritic group was fed the same diet supplemented with 0.4% (w/w) of BAPN. Both groups were fed their respective diets *ad libitum* for 4 weeks. On termination of the experiment demineralized bone matrix was prepared as described earlier (6) from femora and tibiae of control and BAPN-fed rats. Tooth matrix was similarly prepared from mandibular and maxillary incisors. Demineralized bone and tooth matrices, with a particle size of 74–420 μm, were implanted subcutaneously during ether anesthesia into 28-day-old, male, Long–Evans rats (6). The day of implantation was day 0. On Day 11, the alkaline phosphatase activity and ^{45}Ca incorporation in the implants were determined in order to quantitate the osteogenic response as described above.

Histology. Portions of the plaques were fixed in Bouin's fluid and 5-μm sections were cut and stained with toluidine blue.

Materials. L-[1-^{14}C]Ornithine (56 mCi/mmole), (^{35}S) sodium sulfate (960 mCi/mmole), $^{45}CaCl_2$ (18 Ci/g), Aquasol, and Hyamine hydroxide were purchased from New England Nuclear; L-ornithine HCl, EDTA, *p*-nitrophenylphosphate, pyridoxal phosphate, and bovine serum albumin were purchased from Sigma Chemical Company. Dithiothreitol and folin phenol reagents were obtained from Fisher Scientific Company.

Results and discussion. Implantation of demineralized diaphyseal bone matrix to subcutaneous sites in allogeneic rats resulted in sequential differentiation of endochondral bone as described earlier (6–8). A transient inflammation-like response ensued on Day 1 and was followed on Day 3 by mesenchymal cell proliferation in the vicinity of the implanted bone matrix; this was quantitated by ODC activity and revealed a peak on Day 3 (9). On Days 6–8 there was extensive chondrogenesis as indicated by $^{35}SO_4$ incorporation into proteoglycans (10) and the appearance of type II collagen (11). The hypertrophy of the chondrocytes was followed by calcification of the cartilage matrix on Day 9 (6–8). With vascular invasion beginning on Day 9 there was chondrolysis and Days 10–11 were marked by initial osteogenesis as indicated by induction of alkaline phosphatase activity and ^{45}Ca incorporation (8). The BAPN-fed rats exhibited an identical sequence of development as the control rats. However, histologically the amounts of new bone formed was less in the BAPN-fed rats. This was confirmed by the ^{45}Ca incorporation experiments described later. The newly

l bone was remodeled (Days 14–18) matopoietic bone marrow differentia- curred within the ossicle and was max- y Day 21 (7, 8).

enchymal cell proliferation. In our pre- study we demonstrated a peak of ODC y on Day 3, concomitant with extensive ration of mesenchymal cells (9). In the t experiments BAPN-fed rats exhibited activity (303 ± 19 pmole/mg protein/ at was not statistically different from n the controls (278 ± 27 pmole/mg n/hr).

ndrogenesis. The influence of experi- l lathyrism on cartilage differentiation sessed by $^{35}SO_4$ incorporation into pro- cans. As seen in Table I, in Day-7 s there is an inhibition of $^{35}SO_4$ incor- on into the acid-precipitable fraction of glycans. Also noteworthy is that the e phosphatase activity is decreased. inhibitory effects were also seen in the nal tibial epiphyses. The inhibitory ef- $^{35}SO_4$ uptake was confined to the acid- itable fraction in both the plaques and piphyses. This inhibition was probably lated to pool sizes as $^{35}SO_4$ in the acid-soluble fraction in BAPN-fed rats was not different from controls. The present observations on the inhibitory effects of BAPN on chondrogenesis and $^{35}SO_4$ incorporation corroborate the earlier work on chick embryos (12, 13).

Mineralization of cartilage and bone. The influence of BAPN-diet on cartilage calcification and bone mineralization as indicated by alkaline phosphatase activity and ^{45}Ca incorporation is summarized in Table II. On Day 9 during cartilage mineralization lathyrism significantly inhibited both alkaline phosphatase activity and ^{45}Ca incorporation and calcium content. It would appear that there was no delay in the onset of mineralization but the rate of mineralization was impaired. During bone formation and remodeling on Day 14 there was a significant ($P < 0.01$) inhibition of ^{45}Ca incorporation into bone mineral in BAPN-fed rats. In contrast, the alkaline phosphatase levels were maximal in both controls and lathyritic rats on Day 14. The inhibitory effect on ^{45}Ca incorporation in BAPN rats was sustained through Day 21. Alkaline phosphatase and ^{45}Ca incorporation in Day-21 plaques declined in both controls

TABLE I. ALKALINE PHOSPHATASE ACTIVITY AND $^{35}SO_4$ INCORPORATION INTO PROTEOGLYCANS IN DAY 7 PLAQUES

sue	Group	Mean body weight (g)	Alkaline phosphatase (U/mg protein)	$^{35}SO_4$ incorporation (cpm/mg)	
				Acid-soluble supernatant	Acid-insoluble precipitate
	Control	138	0.76 ± 0.13[a]	1309 ± 142	470 ± 87
	BAPN-Fed rats	106	0.31 ± 0.06[b]	1723 ± 150	251 ± 52[b]
ysis	Control		3.0 ± 0.2	1264 ± 61	1207 ± 72
	BAPN-Fed rats		2.3 ± 0.2	1105 ± 49	550 ± 90[b]

an ± SEM of eight observations from four rats.
nificant difference ($P < 0.05$) from control.

BLE II. ALKALINE PHOSPHATASE ACTIVITY AND ^{45}Ca INCORPORATION IN MATRIX-INDUCED PLAQUES

ue	Day	Mean body weight (g)		Alkaline phosphatase (U/mg protein)		^{45}Ca incorporation (cpm/mg tissue)		Calcium content (μg/mg tissue)	
		Control	BAPN	Control	BAPN	Control	BAPN	Control	BAPN
	9	148	114	1.4 ± 0.2[a]	0.5 ± 0.1[b]	910 ± 230	261 ± 96[b]	1.97 ± 0.65	0.22 ± 0.09[b]
	14	153	124	2.2 ± 0.3	2.8 ± 0.4	1410 ± 180	565 ± 75[b]	17.7 ± 3.8	10.3 ± 1.8
	21	152	124	1.5 ± 0.1	1.6 ± 0.3	550 ± 70	304 ± 62[b]	47.2 ± 8.6	19.6 ± 2.7[b]
hysis				6.6 ± 0.6	5.1 ± 0.6	6705 ± 588	3654 ± 462[b]	63.6 ± 2.9	57.8 ± 1.4

an ± SE of eight observations from four rats.
nificant difference ($P < 0.05$) from control.

and BAPN-fed rats. It is noteworthy that the inhibitory effect of BAPN on ^{45}Ca incorporation was also seen in the tibial metaphysis (Table II).

The present results demonstrate that experimental lathyrism was inhibitory to the rate of mineralization in both cartilage and bone. While there was no discernible influence on the onset of mineralization the rate (^{45}Ca incorporation) and extent (calcium content) of this process were profoundly inhibited. While it is attractive to postulate that the deficiency in collagen cross-linking may be responsible for impaired mineralization, it is possible that this effect could in part be due to some unknown metabolic action.

Our results confirm the earlier work (2, 3) on the inhibitory effects of BAPN on mineralization and extend it to discrete stages of endochondral bone mineralization. The present study enabled us to assess the influence of BAPN on *de novo* mineralization of cartilage and bone during development.

Osteoinductive potency of lathyritic bone and tooth matrices. The foregoing results described the influence of BAPN on matrix-induced endochondral bone differentiation. It was of interest to investigate the osteoinductive capacity of normal and lathyritic bone and tooth matrices. Previous studies (14) have demonstrated that bone and tooth matrices from adult rats (1 year old) are equipotent in bone induction. The results are summarized in Table III. The lathyritic bone and tooth matrices are not as active as their normal counterparts in induction of new bone. It is noteworthy that comparison of bone and tooth matrices from 7-week-old rats revealed the latter to be more potent in osteoinduction as measured by alkaline phosphatase activity and ^{45}Ca incorporation. The lathyritic bone matrix was previously demonstrated to be devoid of the inductive signal for bone formation (15). The present observations demonstrate that the lathyritic tooth matrix was as active as the control bone matrix.

TABLE III. COMPARISON OF THE OSTEOINDUCTIVE POTENCY OF BONE AND TOOTH MATRICES OBTAINED FROM CONTROL AND LATHYRITIC RATS IN NORMAL RECIPIENTS

Matrix	Group	Alkaline phosphatase (U/mg protein)	^{45}Ca incorporation (cpm/mg tissue)
Bone	Control	2.2 ± 0.3[a]	2570 ± 356
	Lathyritic	0.2 ± 0.03[b]	45 ± 6[b]
Tooth	Control	5.4 ± 0.5	6330 ± 770
	Lathyritic	4.3 ± 0.5	3375 ± 420[b]

[a] Mean ± SEM of eight observations.
[b] Significant difference ($P < 0.05$) from control.

Summary. The influence of experimental lathyrism generated by β-aminopropionitrile (BAPN) treatment on matrix-induced endochondral ossification was investigated. Although the mesenchymal cell proliferation was not affected, BAPN was inhibitory to chondrogenesis as quantitated by $^{35}SO_4$ incorporation. Both cartilage and bone mineralization, as indicated by ^{45}Ca incorporation, were profoundly inhibited by BAPN treatment. The initial rate and the final extent of mineralization was impaired by the lathyrism. Matrices prepared from lathyritic bone and tooth were not as active as controls in induction of new bone as quantitated by alkaline phosphatase activity and ^{45}Ca incorporation.

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Successful Cold Acclimation Following Bilateral Adrenodemedullation in Rats (40702)

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Although adrenodemedullated rats have been exposed to cold environments (2 to 4°C) for a few hours (1–5) or up to many days (6–9), the contribution of the adrenal medulla in developing cold acclimation remains unclear. Gilgen *et al.* (3) reported that demedullated rats failed to survive in 4°C exposure, dying within 11 hr of exposure. Colonic temperatures were reported to decrease by 4.2°C (1) and 6.1°C (2) after exposure to 4°C environments for 8 or 3 hr, respectively. However, Pouliot (6) found no changes in the colonic temperature of demedullated rats after 6 days in 3°C. Manara *et al.* (4) reported no difference in oxygen uptake between intact and demedullated rats following 1 hr of exposure to 4°C. Conversely, Ring (5) observed a 10% lower oxygen uptake in demedullated than in intact rats acutely exposed to 2–4°C for 3 hr. However, the body temperature of his demedullated rats had fallen below 30°C while the body temperature of intact animals was approximately 33°C. Schönbaum *et al.* (7, 8) have suggested that adrenodemedullated rats given adequate time to permit full regeneration of the adrenal cortex could survive in a cold environment for many weeks and develop the same increased resistance to cold as intact animals. In view of these conflicting results the present study was designed to determine whether adrenodemedullated rats could be successfully cold acclimated (c-a) as indicated by their survival and their calorigenic responses to tyramine and a more intensive cold stimulus of −10°C.

Methods. Twenty-four male Sprague–Dawley rats,[1] each initially weighing 150 g, were randomly divided into two groups. Bilateral adrenodemedullation was performed on one group under sodium pentobarbital (40 mg/kg body wt, ip) anesthesia (10). Three days were allowed for recovery following surgery. Half of the animals in each group were then placed in an environmental chamber at 5°C (±1°C) (c-a), the other rats being maintained at 23°C (±1°C) (non-c-a). All animals were housed in individual cages and Purina Laboratory Chow and water were provided *ad libitum*. A 12-hr light–dark cycle was maintained and food intake and body weight were recorded twice weekly. At the end of the 5-week acclimation period the calorigenic response to an injection of tyramine was determined in all animals. Four or five days later they were exposed to a colder environment (−10°C). Oxygen uptake was measured in a constant-volume, closed system (volume meter, Med Science, model 160) with the rat in a sealed Plexiglas chamber and the expired CO_2 absorbed by soda lime. Two constant-temperature baths were utilized to maintain chamber temperatures of either 28 or −10°C. Colonic and chamber temperatures were continuously recorded on a dual-channel recorder utilizing thermistor probes. Oxygen uptakes and colonic temperatures were first measured at 28°C for 30 min to establish a baseline and to determine the influence of cold acclimation on oxygen uptake and colonic temperature. The animals were then temporarily removed from the chamber, given tyramine–HCl (20 mg/kg body wt, ip) and returned to the chamber for another hour of measurement. Heroux *et al.* (11) had determined that 20 mg/kg of body weight dosage could always elicit a maximum response without apparent toxicity to animals. We were interested in finding a dosage below 20 mg/kg of body weight that might be sufficient in inducing maximal response and determined a tyramine dose–response curve on nine animals using 5, 10, and 20 mg/kg of body weight. However, our finding only served to confirm the observation of Heroux *et al.* Cold tolerance was determined by first

[1] In conducting this research, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee for Laboratory Animal Facilities and Care, of the Institute of Animal Laboratory Resources, National Academy of Sciences–National Research Council.

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measuring oxygen uptakes and colonic temperatures at 28°C and then transferring the animal to the cold bath (−10°C). Measurements of oxygen uptake and colonic temperature were continued for 3 hr or until the animal's colonic temperature reached 17°C. The experimental data were analyzed statistically by a three-factor factorial analysis of variance with repeated measures across time. Where significant interaction effects were found, tests of simple main effects followed. The 0.05 level of significance was selected for rejection of the null hypothesis (12). Since some animals had to be removed before the 3-hr period ended, the analysis of the cold tolerance data was made on the first 100 min of cold exposure. Decreases of colonic temperature in −10°C were analyzed by a series of linear regressions across time to determine the best fitting curve. Significant differences between regression coefficients were determined by the *t*-test.

Results. Removal of the adrenal medulla did not adversely influence growth as indicated by the similarity in mean body weights of the intact and demedullated rats living in either 23 or 5°C. There were no significant differences in either O_2 uptake or colonic temperature measured at 28°C between intact and demedullated rats (Table I). All c-a animals had significantly higher O_2 uptakes ($P < 0.05$) measured at 28°C than the non-c-a animals (Fig. 1, Table I). There was no significant difference in the response of intact or demedullated animals consequent to the cold acclimation period.

All animals had significant increases ($P < 0.01$) in O_2 uptake and colonic temperatures subsequent to the injection of tyramine. In those animals maintained at 23°C, intact rats showed a 55.5% increase in O_2 uptake from 207 to 322 ml/kg 10 min^{-1} while demedullated rats increased 66.8% from 184 to 307 ml/kg 10 min^{-1}. In the c-a groups, intact rats raised their O_2 uptakes by 93.5% (248 to 480 ml/kg 10 min^{-1}) while the demedullated rats showed a 69.7% (234 to 397 ml/kg 10 min^{-1}) increase. Non-c-a rats had 2.1 and 1.7°C increases in colonic temperature while c-a rats exhibited elevations of 3.7 and 3.0°C, respectively, for the intact and demedullated animals. The increases in O_2 uptake and colonic temperature were significantly ($P < 0.01$) greater in c-a animals than in non-c-a animals. There were no significant differences in the increases of O_2 uptake or colonic temperature between the intact and demedullated groups; however, intact c-a rats had significantly ($P < 0.01$) greater increases in O_2 uptake following tyramine than demedullated c-a rats (Fig. 1).

Upon exposure to −10°C there was an immediate increase in oxygen uptake in all rats which attained its highest level some 30 to 40 min after the onset of exposure and then gradually declined. All c-a rats had significantly ($P < 0.05$) higher oxygen uptakes (2720 ml/kg 100 min^{-1} increase over basal uptake in the intact rats and 2863 in the demedullated) than the non-c-a rats (2060 for the intact and 1778 for the demedullated). However, there was no significant difference between the intact and demedullated animals in either the c-a or non-c-a groups (Fig. 2). All animals exhibited a gradual decline in colonic temperature with increased duration of cold exposure. The decline in colonic temperature was significantly ($P < 0.001$) faster non-c-a rats (0.103°C/min for intact and 0.120°C/min for the demedullated) than in the c-a rats (0.054°C/min for the intact and 0.058°C/min for the demedullated). In the non-c-a groups the colonic temperature of demedullated animals decreased at a significantly ($P < 0.05$) faster rate than in intact rats (Fig. 3, Table II).

TABLE I. BODY WEIGHTS, OXYGEN UPTAKES, AND COLONIC TEMPERATURES OF INTACT AND DEMEDULLATED ANIMALS MEASURED AT 28°C FOLLOWING 5 WEEKS OF ACCLIMATION[a]

	Body weight (g)	O_2 Uptake (ml/kg 10 min^{-1})	Colonic temperature (°C)
Non-c-a, intact	405 ± 22.4	207 ± 9.6	36.9 ± 0.2
Non-c-a, demedullated	396 ± 15.6	184 ± 10.2	37.0 ± 0.2
c-a, intact	359 ± 22.8	248 ± 14.7[b]	36.6 ± 0.3
c-a, demedullated	375 ± 11.2	234 ± 7.5[b]	37.1 ± 0.2

[a] Mean ± SE of six animals in each group.
[b] Significantly ($P < 0.05$) higher than corresponding animals maintained at 23°C.

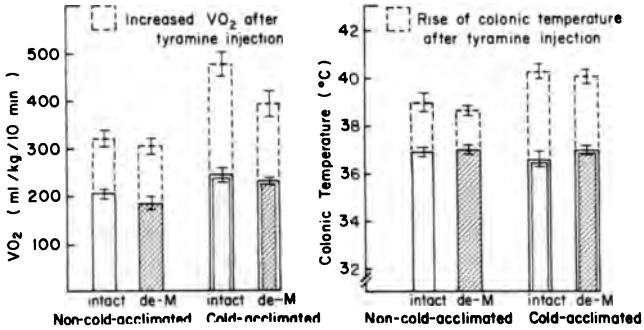
Increased VO_2 after tyramine injection
Rise of colonic temperature after tyramine injection
VO_2 (ml /kg /10 min)
Colonic Temperature (°C)
intact de-M intact de-M
Non-cold-acclimated Cold-acclimated

FIG. 1. Oxygen uptakes and colonic temperatures in response to tyramine: Oxygen uptake (left) and colonic temperature (right) measured at 28°C in intact and demedullated animals acclimated at 5°C (c-a) or 23°C (non-c-a) before and after injection of tyramine. Each bar represents the mean ± SE of values from six rats.

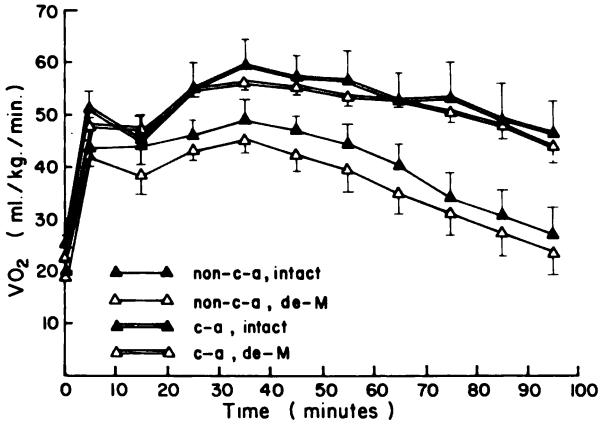

FIG. 2. Oxygen uptakes during first 100 min of exposure to −10°C. Each data point represents average oxygen uptake (ml/kg min^{-1}) in each 10-min period during exposure. Each line represents the mean ± SE of six rats.

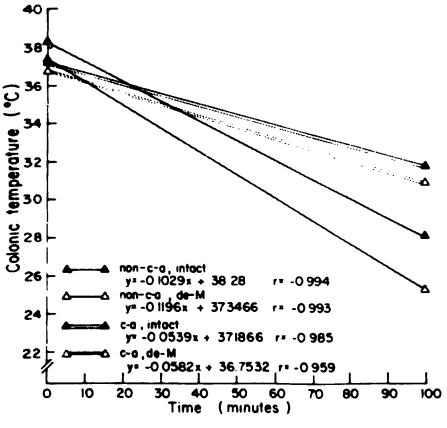
Colonic temperature (°C)
non-c-a, intact
y= -0 1029x + 38 28 r= -0 994
non-c-a, de-M
y= -0 1196x + 373466 r= -0 993
c-a, intact
y= -0 0539x + 371866 r= -0 985
c-a, de-M
y= -0 0582x + 36 7532 r= -0 959
Time (minutes)

FIG. 3. Regression lines of fall of colonic temperature across initial 100 min time in exposure to cold environment of −10°C. Each line represents a group of six rats. The symbols on each line are for the purpose of identification of groups.

TABLE II. COMPARISON OF REGRESSION COEFFICIENTS (β) FOR PREDICTION OF CHANGES IN COLONIC TEMPERATURE IN −10°C WITH TIME (100 min).

	β	t	df	p
Intact vs demedullated				
Intact, non-c-a	−0.1029			
Demedullated, non-c-a	−0.1196	2.8	8	0.05
Intact, c-a	−0.0539			
Demedullated, c-a	−0.0582	0.96	8	n.s.
Non-cold-acclimated vs cold-acclimated				
Non-c-a, intact	−0.1029			
c-a, intact	−0.0539	11.13	8	0.001
Non-c-a, demedullated	−0.1196			
c-a, demedullated	−0.0582	8.5	8	0.001

Discussion. Despite some conflicting evidence that demedullation could result in a failure to survive a cold stress (3), there is

suggestive information that cold acclimation in demedullated animals could occur (6–9). Our data provide convincing evidence that demedullated rats do undergo typical acclimation processes. Pouliot (6) stated that demedullated rats were all normal and normothermic after 6 days of cold exposure. Schönbaum *et al.* (7, 8) observed that demedullated rats after living 2 weeks in 2°C had decreased electromyographic activity in cold compared to non-cold-acclimated, demedullated rats. Jarratt and Nowell (9) noted that the absence of the adrenal medulla did not prevent the usual fluctuations in the blood sugar level observed in intact rats during chronic cold exposure of 130 days. However, the sustained hyperglycemia recorded in prolonged exposure in intact rats was not observed in the demedullated rats.

Evidence that cold acclimation of demedullated rats was similar to that observed in intact rats was shown by: (i) successful survival through 5 weeks of exposure to 5°C, (ii) oxygen uptake measured at 28°C following cold acclimation was raised to the same level observed in intact cold-acclimated animals, (iii) significantly higher increases in oxygen uptake and colonic temperature in response to tyramine administration following cold acclimation although the increase in demedullated animals was less than the increase in intact rats, and (iv) during −10°C exposure increases in oxygen uptake and rates of decrease of colonic temperature were similar to those observed in intact cold-acclimated rats.

During the −10°C exposure colonic temperature decreased at a significantly faster rate in demedullated non-c-a rats than in intact non-c-a rats. In non-cold-acclimated rats the main mode of heat production in cold exposure is shivering thermogenesis. Demedullated animals shiver more vigorously during cold exposure than intact animals (7, 13). The more intense and vigorous shivering in the demedullated animals probably was responsible for the greater heat loss than that seen in intact animals and therefore resulted in a more rapid fall in colonic temperature. Berti *et al.* (1), Maickel *et al.* (2), and Gilgen *et al.* (3), who exposed their intact and demedullated non-cold-acclimated rats to a moderate cold stress of 4°C, also observed that demedullated rats had a more rapid decrease in colonic temperature than did intact rats.

The enhanced calorigenic response to tyramine in c-a rats, intact or demedullated, further confirmed the development of norepinephrine (N-E) thermogenesis and receptor hypersensitivity in cold acclimation. Tyramine acts by releasing endogenous N-E (14). It is not clear whether the enhanced tyramine response in c-a animals is due to increased N-E release or increased sensitivity of β-receptors on the effector cells. The increased β-receptor responsiveness has been elegantly demonstrated by Le Blanc (15) and most recently by Fregly *et al.* (16) in intact c-a rats. There is no direct evidence that tyramine released more N-E in c-a rats although the urinary N-E excretion has been observed to be elevated in these animals (17). However, the smaller increase in oxygen uptake observed in the demedullated than in the intact c-a rats suggested the development of some other adaptive mechanisms. We calculated the heat production and body heat content of both groups of rats before and after the tyramine administration based on body weights, oxygen uptakes, and colonic temperatures. There was no difference in heat production (0.44 kcal/10 min) of the intact or demedullated rats before tyramine, although the mean colonic temperature of the demedullated rats was 0.5°C higher than that of the intact animals (Fig. 1, Table I). Following the tyramine injection intact animals produced more heat than demedullated rats (0.83 kcal/10 min for intact and 0.72 for demedullated; $P < 0.05$). The mean colonic temperatures of both groups after tyramine were similar (40.3 and 40.1°C for the intact and demedullated, respectively). Thus the demedullated rats were not only able to attain a higher colonic temperature than intact rats at comparable levels of heat production prior to tyramine administration, but maintained a similar colonic temperature with a smaller heat production following administration of tyramine. This suggests the possibility that enhanced peripheral vasoconstriction brought about by N-E in the demedullated animals resulted in a lowered heat loss to the environment.

Our results are at variance with the observations of Cottle and Carlson (18). They reported that rectal temperature fell and that

ncreased heat production in demedul- cold-acclimated rats exposed to 5°C was ficantly less than that in the intact ani- . The response to 5°C of their demedul- 28°C acclimated rats did not differ from of their intact 28°C animals. In their rimental protocol, however, the rats were cold acclimated at 5°C, demedullated, then returned to the cold room for ad- nal cold acclimation. Their animals were anesthetized, curarized, and artificially lated when evaluated at 5°C. These dif- ces in experimental design may well ex- . the difference in results obtained.

ır demedullated rats living at 5°C ap- ıtly survived the handicap of not having ephrine from adrenal medulla available em. This may have been a consequence bstitution of other catecholamines in the stage of cold acclimation since increased tion of norepinephrine has been re- d to occur during the first 2 weeks fol- ıg demedullation (19). Immediate in- e of norepinephrine urinary excretion wing cold exposure (2–4°C) of demedul- animals has been observed within the 24 hr (6, 8, 17, 20). Our demedullated may have developed norepinephrine nogenesis sooner than intact rats; how- some epinephrine sources may have available. The urinary epinephrine ex- ɔn by cold-exposed, demedullated rats been reported to be either nonexistent or slightly increased in the cold-exposed the non-cold-exposed animals (6, 8, 19, 2). This slight increase in epinephrine tion in cold-exposed, demedullated an- s may have come from extramedullary maffin cells although there has been no rimental evidence to support this.

ır data indicate that adrenal medullary holamines are not necessary for success- ɔld acclimation but they do not denigrate otential role of epinephrine in the accli- on process. They simply indicate the ty of an organism to meet a stressful tion by utilizing alternative resources.

mmary. Bilaterally adrenodemedullated were successfully cold acclimated at 5°C weeks. This was evidenced by (i) suc- ul survival, (ii) elevated oxygen uptake ured at 28°C, (iii) enhanced calorigenic nse to tyramine although demedullated had significantly lower oxygen uptakes than the intact rats, and (iv) an increased tolerance to cold (−10°C) similar to that observed in intact rats after 5 weeks of acclimation. Non-cold-acclimated demedullated rats were unable to maintain their colonic temperature to the same extent as the intact non-cold-acclimated rats during exposure to −10°C. All cold-acclimated rats had greater response to tyramine and better tolerance to −10°C than non-cold-acclimated rats.

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Serum LH and FSH Levels in the Pregnant Rabbit (40703)

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Introduction. Early work in the rabbit, an induced ovulator, demonstrated that coital stimulation triggers a release of gonadotropin from the pituitary (1, 2). More recently, workers have shown that a sharp rise in serum luteinizing hormone (LH) concentration takes place as early as 15 min after mating with peak values by 2 hr and subsequently returns to precoital levels by 4 to 6 hr (3–7). Dufy-Barbe *et al.* (4) reported no concomitant increase in serum levels of follicle stimulating hormone (FSH) through the first 4 hr postcoitum. In contrast, Goodman and Neill (6) did report a significant rise in serum FSH by 2 to 3 hr after mating with a slightly elevated FSH value at 5 hr. Differences in assay sensitivity may possibly account for this discrepancy since the first group of workers (4) used a heterologous gonadotropin assay system (ovine-LH as standard), while the latter workers (6) utilized a system designed specifically for determination of rabbit gonadotropin levels. Despite these published studies of gonadotropin levels in the preovulatory period, little has been reported concerning the levels of these hormones during pregnancy in the rabbit. Scaramuzzi *et al.* (3) measured serum LH in pregnant rabbits at selected time periods and found that serum LH levels were not different from those found in the precoital period. Simultaneous measurements of both LH and FSH in the serum of rabbits throughout the entire period of pregnancy have not been reported.

As a part of ongoing studies from this laboratory on the function of the rabbit ovary, we have examined the levels of serum LH and FSH through pregnancy. We sought to define the gonadotropic support available to the ovary, especially over the first 5 postcoital days. During this time, the ovary is transformed from an estrogen-producing organ dominated by large follicles to a structure containing numerous corpora lutea synthesizing principally progesterone.

Materials and Methods. Animals. Sexually mature male and female white rabbits of the New Zealand strain (3.5–4.5 kg) were obtained from a local supplier and kept isolated from one another. Rabbits were maintained at constant temperature on a 14-hr light, 10-hr dark schedule with pressed food and water *ad libitum.*

Mating was accomplished by placing the doe in the cage of a male rabbit. The success of matings was confirmed by microscopic examination of vaginal washings for the presence of sperm.

Blood Samples. Sequential peripheral venous blood samples were drawn from the marginal ear vein of each rabbit immediately before and 1, 2, 3, 4, and 12 hr after mating. Additional samples were collected on Day 1 (24 hr after mating) and Days 2, 3, 4, 5, 8, 11, 14, 18, 21, 24, 27, and 31. These samples were allowed to clot on ice and serum collected centrifugally at 4°C. All serum samples were stored at −20°C until assayed for gonadotropin.

Gonadotropin Assay. Serum LH and FSH were measured in each serum sample by means of specific homologous double-antibody radioimmunoassays utilizing NIAMDD kits for rabbit gonadotropin. These kits included purified gonadotropin (NIAMDD-LH-AFP-559-B and NIAMDD-FSH-AFP-538-C) which were used both as standards and for iodination.

Radioiodination procedure. The purified LH and FSH preparations were radiolabeled with ^{131}I according to the chloramine-T procedure of Greenwood *et al.* (8). Labeled hormone was separated from unbound iodine on a column of Bio-Gel P-60. The radioactive hormone was diluted in 0.1% bovine serum albumin phosphate-buffered saline (BSA-PBS) at a concentration of 2 to 3 ng/100 μl containing 30,000 to 40,000 cpm.

Antisera. Antibodies against rabbit LH (AFP-8-1-28) and against rabbit FSH (AFP-2-7-1), had been raised in guinea pigs and were supplied with the kits. The anti-LH was

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d to 1:600,000 for use in the assay while ıti-FSH was diluted to 1:60,000. In both , the hormone–antibody complex was ıitated with a 1:200 dilution of anti- ı pig γ-globulin raised in goats.

ay procedure. The assays were per- d over a 7-day period at 4°C. Dupli- of 200-μl serum samples were diluted to with 1% BSA–PBS and then incubated hr in the presence of the specific anti- The respective labeled hormones were ıdded in 100 μl of 0.1% BSA–PBS fol- by another 24-hr incubation. The sec- ntibody was then introduced in a vol- of 200 μl and the incubation continued additional 4 days. After centrifugation, pernatant fractions were discarded and I content of the precipitates counted in ckman gamma counter (model 310). ard lines ran from 0.0625 to 4 ng for ıd from 0.0625 to 5 ng for FSH.

e average intraassay coefficient of vari- was 6.8% for the duplicate samples. tical analyses were performed using an's multirange test (9).

ults. Five mated rabbits delivered nor- tters (average litter size 10.2) from 31 to ys after mating. All does prepared sat- ory nests and exhibited normal nursing ior.

e pattern of LH in serum through the days of pregnancy is shown in Fig. 1. tistically significant increase in serum as seen at 1 hr after mating with peak s by 2 hr ($P < 0.05$). The serum LH then ed continuously, returning to estrous by 12 hr postcoitum. No further in- e in serum LH was seen through the 5 days of pregnancy in any of the does. e pattern of FSH in serum is shown gh the first 5 days of pregnancy in Fig. ough considerable animal variation was nt, a significant increase in serum FSH bserved by 1 hr after mating ($P < 0.05$). values reached a maximum at 2 to 3 hr oitum and then declined to precoital by 12 hr. In contrast to serum LH, a d statistically significant surge of FSH red ($P < 0.05$), reached a maximum at d 48 hr postcoitum, and then declined ecoital levels by Day 3 of pregnancy. econd serum peak of FSH showed con- ıbly less animal variation than the first FSH surge and was exhibited by all five pregnant animals.

The serum LH and FSH levels at various days through the duration of pregnancy are shown in Table I. No significant increase in the serum concentrations of either gonadotropin was seen from Day 8 of pregnancy to delivery.

Discussion. Many species, including the rabbit, are dependent upon pituitary gonadotropin support of the ovary throughout pregnancy. We have used homologous kits for rabbit gonadotropins supplied by the NIAMDD to measure serum LH and FSH throughout the duration of pregnancy in the species.

Serum LH rose from an estrous value of 0.6 ± 0.14 to 12.6 ± 1.8 ng/ml by 2 hr

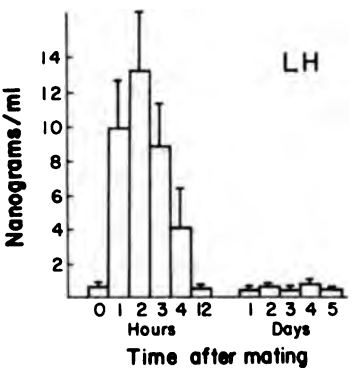

FIG. 1. Serum LH levels before and at various times after mating. Each bar represents the mean of duplicate determinations performed on sera from five different does. Brackets represent one SEM.

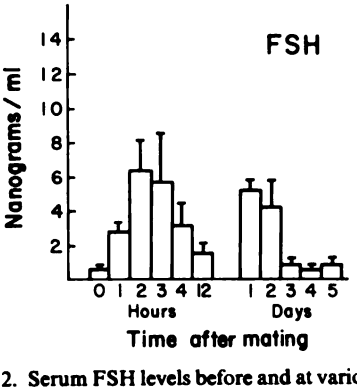

FIG. 2. Serum FSH levels before and at various times after mating. Each bar represents the mean of duplicate determinations performed on sera from five different does. Brackets represent one SEM.

TABLE I. SERUM LEVELS OF LH AND FSH AT VARIOUS DAYS THROUGH PREGNANCY IN THE RABBIT.[a]

Day of pregnancy	LH (ng/ml)	FSH (ng/ml)
8	0.63 ± 0.12	1.06 ± 0.08
11	0.74 ± 0.14	1.27 ± 0.08
14	0.45 ± 0.04	1.25 ± 0.10
18	0.41 ± 0.02	0.82 ± 0.08
21	0.42 ± 0.03	0.82 ± 0.07
24[b]	0.52 ± 0.08	1.4 ± 0.1
27	0.45 ± 0.04	1.4 ± 0.11
31[c]	0.42 ± 0.01	0.83 ± 0.16

[a] Values reported are mean ± SEM for sera from five animals.
[b] Represents sera from two animals.
[c] Represents sera from three animals.

postcoitum and gradually returned to precoital levels by 12 hr. This general pattern of LH release with mating has been reported by several investigators (3–7). Each of the pregnant animals in this study exhibited a similar rapid rise in serum LH through the preovulatory period. Measurements of serum LH at various days of pregnancy showed no further significant increases above the 12-hr level. This finding is in agreement with the study of Scaramuzzi *et al.* (3) in which pregnant rabbits at 14–15 or 24–25 days of pregnancy showed no elevated serum LH levels. Therefore, only tonic, but measurable levels of LH appear to be necessary for the maintenance of pregnancy in the rabbit.

Serum FSH values also exhibited a preovulatory surge in three of the five animals studied, rising from an estrous value of 0.6 ± 0.07 to 6.1 ± 1.4 ng/ml at 2–3 hr postcoitum. Serum FSH values then decreased to nearly precoital values at 12 hr after mating. The magnitude, duration, and animal variability seen in this FSH peak is similar to that reported by Goodman and Neill (6), who used a similar homologous FSH assay system. Following the preovulatory FSH peak, a second surge of FSH occurred at a period when serum LH was not elevated. A similar second peak of FSH, distinct from LH, has been shown to occur in rodents. Most recently, Chappel *et al.* (10), working with hamsters, demonstrated this rise of FSH on the first day of estrus after the proestrus surges of FSH and LH had returned to basal concentrations.

The significance of the postovulatory FSH surge in the rabbit may lie in the control of follicular development during this critical period of pregnancy or pseudopregnancy. Postovulatory follicle development in the rabbit is of special interest since the corpus luteum in this species is an estrogen-dependent tissue (11, 12). Specific receptors for estradiol-17β have been reported to be present in 3- to 4-day-old corpora lutea (13) with total estrogen dependence 1–2 days thereafter (14). The source of the luteotropic estrogen appears to be the large follicles present during this time (12). The growth and development of these "luteotropic follicles" to replace those which have ovulated may be the target of the postovulatory FSH surge.

Summary. Serum levels of LH and FSH were measured in the pregnant rabbit utilizing homologous assays for rabbit gonadotropins. Results show that following mating, serum LH rose to peak levels by 2 hr postcoitum, returned to estrous levels by 12 hr, and remained at low levels through the duration of pregnancy. Serum FSH also exhibited a preovulatory surge at 2–3 hr after mating, returning to precoital levels by 12 hr. In contrast to serum LH, however, a second surge of serum FSH occurred at 1–2 days of pregnancy at a time when serum LH was not elevated. Following the second peak, serum FSH also remained low through the remainder of pregnancy.

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Effect of Intravenous Fat Emulsion on Pentobarbital-Induced Sleep Time in Rats (40704)

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The introduction of Intralipid, a 10% soybean oil emulsion, represented a major clinical advance in parenteral nutrition in that it provides large amounts of energy in relatively small volumes (1). The intravenous administration of fat emulsion appears to be safe and free of serious risks or complications (2–4). However, with intravenous fat emulsion therapy, because of the lipophilic nature of many drugs there is a potential for drug interactions. In order to test this possibility, we examined whether the anesthetic action of the lipid-soluble drug sodium pentobarbital might be altered by the intravenous administration of 10% fat emulsion. We also determined whether the metabolic depressant action of pentobarbital would affect the clearance of the fat emulsion from the plasma.

Materials and methods. Sleeping times. Male Sprague–Dawley rats weighing 300–350 g were used in all experiments. The animals were given food (Purina Rat Chow) and water *ad libitum*. While the animals were under ether anesthesia, a saline-filled PE-50 cannula was placed in the right jugular vein and exteriorized through the skin at the back of the neck. Each animal was allowed 90 min recovery time. Narcosis was then induced by intravenous administration of pentobarbital (30 mg/kg) and the time (minutes) to the return of the righting reflex was determined. When Intralipid emulsion (10%, Cutter Laboratories) was given, it was infused through the venous cannula over 30 sec. A saline volume control was employed for comparison with all doses of Intralipid emulsion tested. Control rats were included with each group of experimental rats in order to control for differences related to time of day or day-to-day variation.

Intralipid emulsion clearance. Our observations on the effect of Intralipid emulsion on pentobarbital-induced sleep times suggested that clearance of the administered fat emulsion from the plasma may be an important determinant of the interaction observed. We, therefore, determined the clearance and half-life of Intralipid emulsion from the plasma according to the procedure described by Carlson and Rössner (5). While the animals were under ether anesthesia, saline-filled cannulas were placed in the jugular vein and carotid artery. Both of these cannulas were exteriorized through the skin at the back of the neck. The rat was placed in a restrainer and allowed 90 min for full recovery. A bolus injection of 10% Intralipid emulsion in a dose of 0.2 g per kilogram of body weight was given via the jugular vein over a period of 15 sec. At time intervals of 0, 2, 5, 10, 20, 25, and 30 min after infusion of the emulsion, 0.2 ml of blood was taken from the carotid artery and placed in 5.0 ml of an isotonic saline solution. The blood samples were centrifuged immediately at room temperature at 700 rpm for 10 min to sediment red blood cells. Two milliliters of the supernatant was placed in culture tubes (10 × 75 mm), and the molecular light scattering index was determined with a PDQ® Laser Nephelometer (Hyland Diagnostics Division of Travenol Laboratories, Inc., Costa Mesa, Calif.). A standard curve with Intralipid emulsion in saline was used to convert the molecular light scattering index values into milligrams of triglyceride per 100 ml of plasma. Zero-time nephelometry reading was subtracted to correct for endogenous light scattering particles. At this dose the disappearance of Intralipid emulsion from the plasma of rats follows first-order kinetics in which removal is an exponential function (6). The log of the plasma Intralipid emulsion concentration (mg/100 ml) plotted against time gives a straight line. The slope of this line by convention defines the rate constant

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first-order reaction rate and is ex-
as a percentage removed per minute
he biologic half-life ($t_{1/2}$) in minutes
culated using the formula $t_{1/2} = 0.693/$
values for K_2 and $t_{1/2}$ were obtained
tandard programs using a Texas In-
nts 59 calculator. Data are expressed
n ± standard error, and the differences
n means were analyzed using one-way
s of variance and least significant dif-
test of means (7).

lts. Sleeping time. In our initial studies,
0% Intralipid emulsion (2.5 g/kg, iv)
ministered 5 min after pentobarbital-
l narcosis, sleep time (minutes) was
ed considerably, 87 ± 6 (n = 15) vs
15 (n = 6), $P < 0.001$. As the dose of
id emulsion was decreased to 0.16 g/
prolongation of sleeping time was also
ed in a dose-dependent fashion (Fig.
en Intralipid emulsion (2.5 g/kg, iv)
ven 5 min prior to pentobarbital-in-
narcosis, a significant ($P < 0.001$),
h slightly smaller prolongation of
g time was observed, 87 ± 6 (n = 15)
± 14 (n = 6).

26-min difference between the mean
g times of rats given Intralipid emul-
min before and those given it 5 min
pentobarbital-induced narcosis sug-
the importance of clearance of the fat
on from the plasma as a factor in the
gation of pentobarbital-induced sleep-
e. When the time of administration of
id emulsion was increased to 60 min
pentobarbital anesthesia, the prolon-
of sleeping time was reduced in a time-
lent fashion (Fig. 2). If the amount of
id emulsion present in the plasma at
en time is causally related to the pro-
on of pentobarbital-induced sleep time
eparin, an agent demonstrated to in-
the clearance of triglycerides from the
(8), would be expected to decrease the
gation of sleep time at any particular
f Intralipid emulsion. Results testing
pothesis are shown in Table I. Heparin
nits/kg, iv) significantly ($P < 0.01$)
ed the prolongation of sleep time at
o doses of Intralipid emulsion tested.
e the Intralipid emulsion was admin-
while the animals were in three differ-
tes (conscious, pentobarbital-anesthe-
tized, and heparinized), we examined the clearance and half-life of Intralipid emulsion under these various conditions. The effect of pentobarbital and heparin on Intralipid emulsion clearance is shown in Fig. 3. There was a significant difference ($P < 0.01$) among the pentobarbital-anesthetized, heparinized, and conscious animals in the percentage removal (K_2) of Intralipid emulsion, with heparin augmenting the clearance rate and pentobarbital decreasing it. These values are summarized in Table II, along with the $t_{1/2}$ values and the coefficients of correlation describing the respective straight lines. It can be seen that heparin increases the clearance of Intralipid emulsion from the plasma and decreases the half-life, while pentobarbital decreases the clearance of Intralipid emulsion

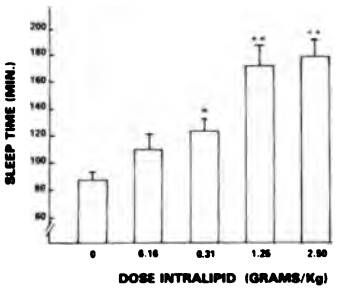

FIG. 1. Effect of Intralipid emulsion (iv) on pentobarbital (30 mg/kg)-induced sleep time. Intralipid emulsion was administered 5 min after pentobarbital anesthesia and the time to the return of the righting reflex was determined. Values are the means ± SEM of six animals at each dose except control (0 Intralipid emulsion) where n = 15. $^*P < 0.05$, $^{**}P < 0.001$ by group comparison with control values.

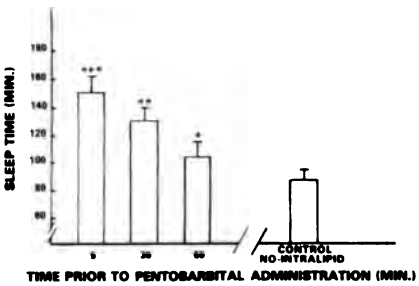

FIG. 2. Effect of Intralipid emulsion (2.5 g/kg, iv) on pentobarbital (30 mg/kg)-induced sleep time. Intralipid emulsion was administered at 5, 30, and 60 min before pentobarbital anesthesia and the time to the return of the righting reflex was determined. Values are the means ± SEM of six animals at each time. $^{***}P < 0.001$, $^{**}P < 0.005$, and $^*P < 0.05$ vs control values.

and increases the half-life.

Discussion. Pentobarbital sleep time in the rat was prolonged in a dose-dependent fashion by the administration of 10% Intralipid emulsion. Although the doses employed in this study were high compared to clinical dosage (4), the high metabolic rate of the rat must be taken into account. The rat utilizes oxygen at about 20 ml/min/kg (9) and man at about 3.5 ml/min/kg (10). In addition, in fasted man, the clearance of Intralipid emulsion following a bolus injection (0.1 g/kg) was reported to be approximately 4%/min (11–14) while we observed a 16.6%/min clearance in the conscious rat.

The mechanism whereby Intralipid emul-

TABLE I. EFFECT OF HEPARIN (200 units/kg) ON THE PROLONGATION OF PENTOBARBITAL-INDUCED SLEEP TIME BY INTRALIPID EMULSION.[a]

Treatment	Sleep time (min)
Control	87 ± 6
Intralipid (2.5 g/kg)[b]	131 ± 10[c]
Intralipid (2.5 g/kg) + heparin[b]	90 ± 5[d]
Intralipid (1.25 g/kg)[e]	169 ± 16[c]
Intralipid (1.25 g/kg) + heparin[e]	112 ± 8[d]

[a] Values are the means ± SEM of six animals with each treatment except control where $n = 15$.
[b] Thirty minutes prior to pentobarbital anesthesia.
[c] $P < 0.01$ vs control value.
[d] $P < 0.01$ vs corresponding Intralipid emulsion (without heparin) value.
[e] Five minutes after pentobarbital anesthesia.

TABLE II. EFFECT OF HEPARIN AND PENTOBARBITAL ON THE FRACTIONAL REMOVAL RATE (K_2) AND HALF-LIFE ($t_{1/2}$) OF INFUSED INTRALIPID EMULSION (0.2 g/kg).[a]

	Control	Heparin (200 units/kg)	Pentobarbital (30 mg/kg)
K_2[b]	16.6 ± 1.6	37.2 ± 3.0[c]	8.7 ± 1.2[c]
$t_{1/2}$[d]	4.3 ± 0.4	1.84 ± 0.2[c]	8.2 ± 0.9[c]
r[e]	0.980	0.980	0.970
n	14	6	7

[a] Values are the means ± SEM.
[b] K_2 expressed as percentage removed/minute.
[c] $P < 0.01$ vs control values.
[d] $t_{1/2}$ was calculated from $0.693/K_2$.
[e] r = correlation coefficient describing fit to a first-order reaction rate.

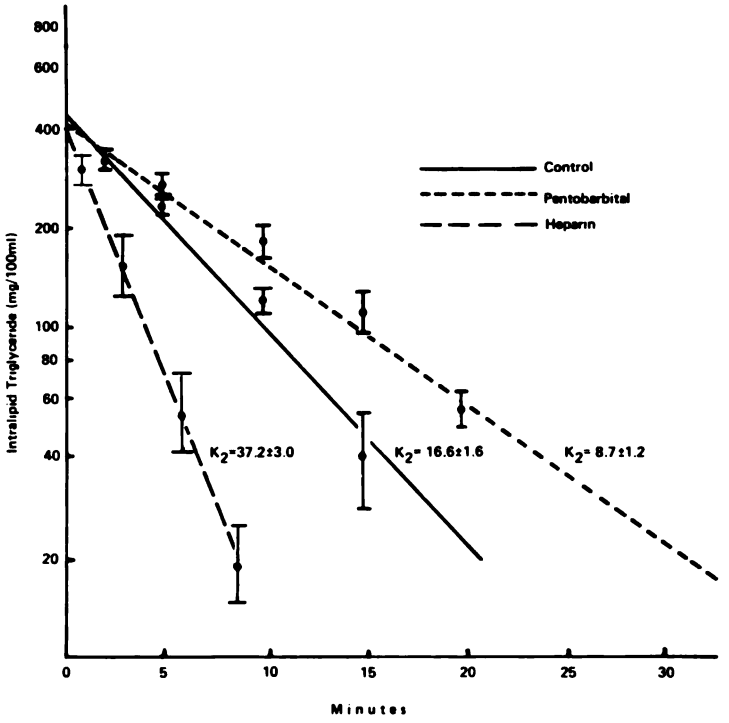

FIG. 3. Effect of heparin (200 units/kg, iv) and pentobarbital (30 mg/kg, iv) on fractional removal rate (K_2) of infused Intralipid emulsion (0.2 g/kg). Each point is the mean ± SEM. Animals in each group were as follows: control ($n = 14$), heparin ($n = 6$), and pentobarbital ($n = 7$).

prolongs sleep time is not known at nt, but several possibilities exist. Plasma cerides or chylomicrons increase after injection of fat emulsion. This plasma of lipid may serve as a depot for pento- tal just as tissue lipids do. Fat emulsion e blood may take up pentobarbital be- : of its lipophilic nature. As long as)barbital is contained or trapped within pid emulsion, it may not be available for bolism by the liver. Likewise, pentobar- in the lipid emulsion would not be avail- to exert an anesthetic effect. Conse- tly, although the duration of anesthesia be prolonged, the depth of anesthesia be reduced. Heparin, by increasing the ance of Intralipid emulsion from the na, may free the "protected" pentobar- and accelerate its metabolic degrada- thus decreasing its pharmacologic effect. ice lipid solubility is an important deter- nt of whether a drug is metabolized by icrosomal enzyme system, fat emulsion competitively inhibit the microsomal -metabolizing enzyme system when ad- stered in very high doses. A drug inter- n may occur between lipid-soluble com- ds because one may competitively in- the metabolism of the other. It has been onstrated that ethyl alcohol, which is not ally metabolized by the microsomal sys- can inhibit drug hydroxylations when nistered acutely (15). In the same study s postulated that high concentrations of alcohol in liver cells competitively in- the microsomal hydroxylase system. e the acute administration of alcohol eases pentobarbital metabolism, chronic inistration increased it (16). There are y compounds that inhibit the metabolism drug when given acutely and stimulate etabolism of the same drug after chronic inistration (17, 18). Although it is not vn whether Intralipid emulsion under rent modes of administration would have : varying effects on microsomal function, s been demonstrated that chronic admin- ion of fat in the diet can augment micro- l drug metabolism. Caster *et al.* (19) rted that, as the intake of corn oil was ased to 3% of the diet, there was an ase in the ability of the liver, in an *in* system, to metabolize drugs. Increases in drug microsomal metabolism and in the amount of cytochrome *P*-450 in the hepatic microsomes of rats fed high levels of corn oil were also reported by Norred and Wade (20). Other workers (21, 22) have shown that the rate of microsomal metabolism of various drugs is increased when rats are fed diets containing polyunsaturated as opposed to more saturated fats. Whether chronic administration of Intralipid emulsion would augment microsomal metabolism of drugs, in a manner similar to the way in which chronic dietary administration of fat does, remains to be determined.

Another possible explanation for the prolonged sleep times may be that free fatty acids generated by the breakdown of fat emulsion inhibit hepatic microsomal metabolism of pentobarbital. Di Augustine and Fouts (23) demonstrated *in vitro* that unsaturated fatty acids competitively inhibit microsomal O-demethylation and N-demethylation.

Since the prolongation of sleep time was found to be related to both the dosage of Intralipid emulsion (Fig. 1) and the time of administration (Fig. 2), the concentration of Intralipid emulsion in the plasma at any given time appears to be an important determinant of its interaction with pentobarbital. Thus the time-dependent nature of Intralipid emulsion's ability to prolong sleep time may be related to the amount of fat removed from the plasma which increases as the time between administration of the emulsion and the time of pentobarbital anesthesia increases from 5 to 60 min. This is further supported by the fact that the prolongation of sleep time at various dosages of Intralipid emulsion decreases following the administration of heparin (Table I). We (Fig. 3) and others (7) have shown that heparin increases the clearance and decreases the half-life of Intralipid emulsion in the blood.

The mechanism by which pentobarbital delays the removal of Intralipid emulsion from the blood is not clear. Similar results were obtained using chylomicrons labeled with [^{14}C]palmitic acid (24). Heparin augments the clearance of Intralipid emulsion from the plasma by releasing the lipoprotein lipase that is present at the luminal surface of the capillary endothelium in various extrahepatic tissues (25). Lipoprotein lipase pro-

motes the breakdown of triglycerides present in fat emulsion, so that its fatty acid constituents can be oxidized or stored. This enzyme is the major pathway for assimilation of intravenously administered lipid and is extremely sensitive to various physiological and pharmacological perturbations (26). Whether pentobarbital can depress lipoprotein lipase activity is not known at present. Mallov and Cerra (27) could demonstrate no effect on cardiac lipoprotein lipase by prolonged sodium pentobarbital anesthesia.

A significant amount of the total lipoprotein lipase in the rat is found in skeletal muscle (28), and a blood flow reduction to this area may decrease the rate of interaction between enzyme and substrate and result in the decreased removal of Intralipid emulsion from the blood. However, this mechanism does not explain the action of pentobarbital on fat emulsion clearance from the plasma since pentobarbital anesthesia does not alter blood flow to skeletal muscle (29).

It has also been demonstrated that pentobarbital anesthesia depresses total body oxygen consumption and this decrease is largely the result of decreased skeletal muscle metabolism (30). It is possible that lipoprotein lipase activity in skeletal muscle is reduced secondary to this effect, resulting in a decreased removal rate of Intralipid emulsion from the blood.

Whatever the mechanism, since pentobarbital decreases the clearance of Intralipid emulsion, it may, in turn, enhance Intralipid emulsion's effect on pentobarbital-induced narcosis. Thus, these studies have demonstrated a reciprocal interaction between Intralipid emulsion and pentobarbital, with Intralipid emulsion prolonging pentobarbital anesthesia, and pentobarbital anesthesia decreasing the clearance of Intralipid emulsion.

Summary. A 10% soybean oil emulsion (Intralipid) when given intravenously was found to prolong pentobarbital-induced sleep time in rats. The prolongation of sleep time was dose and time dependent. Heparin increased the clearance of Intralipid emulsion from the plasma and decreased the amount of time by which sleep was prolonged. It was further demonstrated that pentobarbital anesthesia decreased the clearance of intravenously administered Intralipid emulsion from the plasma. Thus, a reciprocal interaction was observed whereby pentobarbital prolonged the half-life of Intralipid emulsion and Intralipid emulsion prolonged the narcosis induced by pentobarbital.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 464–466 (1979)

Effect of Zinc Ions on the Actions of Prolactin on RNA and Casein Syntheses in Mouse Mammary Gland Explants (40705)[1]

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Introduction. We have demonstrated earlier that cyclic GMP imitates the stimulatory action of prolactin on RNA synthesis in cultured mouse mammary gland explants (1). Deviller *et al.* (2) have shown that zinc is a potent inhibitor of guanylate cyclase activity in homogenates of lymphocytes. The present studies were therefore designed to test whether zinc ions would affect guanylate cyclase activity in homogenates of mouse mammary tissues. In addition, the actions of prolactin and insulin on RNA and casein synthesis in cultured mouse mammary tissues were tested with zinc ions added to the culture medium.

Materials and methods. Pregnant Swiss-webster mice were used for experimentation 10–14 days after Day 1 of pregnancy; they were purchased from Spartan Research Animals Inc., Haslett, Michigan. Ovine prolactin (NIH-P-S-13) was obtained from the National Institute of Arthritis and Metabolic Diseases. Other substances were purchased from the following sources: hydrocortisone from Charles Pfizer and Company; Nystatin from E. R. Squibb and Sons, Inc.; Medium 199, penicillin, and streptomycin from KC Biological Inc.; [5,6-^{3}H]uridine (41.3 Ci/mmol), [4,5-^{3}H]leucine (51–56 Ci/mmol), guanosine 5′-[α-^{32}P]triphosphate (GTP), and [^{3}H]guanosine 3′,5′-cyclic monophosphate (cyclic GMP) from the New England Nuclear Corporation; porcine insulin from the Eli Lilly Company; GTP and cyclic GMP from the Sigma Chemical Company.

Incubations and preparation of mammary gland explants were accomplished by methods employed earlier (3). Briefly, explants (3–5 mg each) were prepared for incubation and three explants were placed on siliconized lens paper floating on 2 ml Medium 199. When the effect of zinc on the insulin stimulation of RNA synthesis was to be studied, the tissues were cultured for 24 hr in the absence of hormones. Insulin (2.5 μg/ml) and/or zinc were then added to the culture medium and incubation was continued for 4 hr. [^{3}H]Uridine was added to the culture medium for the final hour of incubation. The amount of [^{3}H]uridine incorporated into RNA was then assessed as described earlier (3). If the effects of zinc on the prolactin stimulation of RNA and casein syntheses were to be studied, the explants were initially cultured for 2 days with Medium 199 containing insulin (2.5 μg/ml) and hydrocortisone (1 μg/ml). Prolactin (2.5 μg/ml) and/or zinc were then added to the culture medium and incubations were continued for 4 hr (RNA experiments) or 14 hr (casein experiments). The rates of [^{3}H]-uridine incorporation into RNA and [^{3}H]leucine incorporation into a casein-rich protein fraction were used as indices of the rates of RNA and casein syntheses, respectively; these labeled compounds were added to the culture media (1 μCi/ml) 1 hr prior to termination of the incubations. Methods used to isolate and quantitate the labeled macromolecules were described earlier (3). Zinc was added to the culture medium as the chloride salt.

Guanylate cyclase activity in mammary gland homogenates was assessed by the rate of [α-^{32}P]GTP conversion to cyclic [^{32}P]GMP as was described earlier (4). Briefly, tissues were homogenized 1:10 (w/v) and incubated for 15 min at 30°C with 0.1 m*M* [^{32}P]GTP (0.5 μCi/tube). The cyclic GMP formed during the reaction was isolated by ion exchange chromatography and salt precipitation with $Ba(OH)_2$ and $ZnSO_4$. The cyclic GMP formed per 10 mg tissue was then calculated.

Statistical comparisons were made with Student's *t* test or the multiple *t* test where appropriate.

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ts. Table I shows that zinc ion con-
ons of 0.025 m*M* and above abolished
lactin stimulation of [^{3}H]uridine in-
tion into RNA. In addition, 1 m*M*
uced by more than 75% the basal rate
ıridine incorporation. Zinc ion con-
ons of 0.5 m*M* and above similarly
d the action of prolactin on the rate
ucine incorporation into casein (Ta-
Zinc, by itself, caused a concentration-
nt inhibition of the rate of [^{3}H]leu-
orporation. Table III shows the effects
us zinc ion concentrations on guanyl-
ase activity in homogenates of mam-
ısues. Zinc ion concentrations of 0.05
d above inhibited guanylate cyclase
in a concentration-dependent man-
ıally, the effect of zinc ions on the
stimulation of RNA synthesis in cul-
ammary tissues was tested (Table IV).
ntration-dependent inhibition of in-
ction on RNA synthesis was observed
inc ion concentration was increased.
0.1 m*M* zinc ion concentration, the
response was no longer significant.

ssion. These studies show that zinc
s the actions of prolactin on RNA
ein syntheses in mouse mammary

BLE I. EFFECT OF VARIOUS ZINC ION
RATIONS ON THE PROLACTIN STIMULATION OF
RNA SYNTHESIS.[a]

oncen-on)	[^{3}H]Uridine incorporation into RNA (dpm/μg RNA)		
	Control	Prolactin	*P*
ıent I			
	171 ± 5	249 ± 11	<0.01
10^{-3}	39 ± 4	33 ± 3	N.S.
10^{-5}	140 ± 8	132 ± 7	N.S.
10^{-6}	181 ± 10	180 ± 13	N.S.
10^{-5}	190 ± 13	271 ± 10	<0.01
ıent II			
	180 ± 11	320 ± 20	<0.01
10^{-5}	170 ± 10	324 ± 8	<0.01
10^{-6}	158 ± 11	332 ± 16	<0.01
10^{-7}	168 ± 10	322 ± 14	<0.01
10^{-7}	165 ± 10	320 ± 22	<0.01

ants were cultured for 2 days with insulin plus
lisone. Prolactin and/or zinc were then added
flasks and incubation was continued for 4 hr.
ne, 1 μCi/ml, was added to the medium 1 hr
ermination of the incubations. Numbers repre-
neans ± standard errors of explants from seven

TABLE II. EFFECT OF VARIOUS ZINC ION CONCENTRATIONS ON THE PROLACTIN STIMULATION OF CASEIN SYNTHESIS.[a]

$ZnCl_2$ concentration (*M*)	[^{3}H]Leucine incorporation into casein (dpm/mg tissue)		
	Control	Prolactin	*P*
Experiment I			
0	380 ± 14	630 ± 37	<0.01
1×10^{-4}	145 ± 10	154 ± 9	N.S.
5×10^{-5}	257 ± 9	266 ± 18	N.S.
2.5×10^{-5}	350 ± 16	442 ± 13	<0.01
1×10^{-5}	407 ± 22	760 ± 47	<0.01
Experiment II			
0	532 ± 29	877 ± 21	<0.01
1×10^{-5}	490 ± 32	787 ± 32	<0.01
1×10^{-6}	471 ± 17	795 ± 44	<0.01
4×10^{-7}	477 ± 21	796 ± 19	<0.01
1×10^{-7}	522 ± 24	830 ± 25	<0.01

[a] Explants were cultured for 2 days with insulin plus hydrocortisone. Prolactin and/or zinc were then added to certain flasks and incubation was continued for 14 hr. [^{3}H]Leucine, 1 μCi/ml, was added to the medium 1 hr prior to termination of the incubations. Numbers represent the mean ± standard error of explants from seven flasks.

TABLE III. EFFECT OF VARIOUS CONCENTRATIONS OF ZINC ON GUANYLATE CYCLASE ACTIVITY IN HOMOGENATES OF MOUSE MAMMARY TISSUES.[a]

$ZnCl_2$ concentration (m*M*)	Guanylate cyclase activity (pmol/15 min/10 mg)
1	30.6 ± 6.2
0.5	38.0 ± 2.8
0.2	60.9 ± 3.6
0.1	72.1 ± 2.1
0.05	83.5 ± 3.5
0.01	89.8 ± 1.9
0.005	87.9 ± 2.1
0	93.5 ± 5.1

[a] Guanylate cylase activity in 1:10 (w/v) tissue homogenates was measured as described in the Materials and Methods section. Numbers represent the mean ± standard error of quadruplicate determinations.

gland explants. These inhibitory actions of zinc ions occurred at zinc concentrations which inhibited guanylate cyclase activity in broken cell preparations of mammary glands. It was postulated earlier that because cyclic GMP mimics the action of prolactin on RNA synthesis in the mammary gland (1), cyclic GMP may be involved in certain of the actions of prolactin in mammary tissues. Compatible with this hypothesis were the observations that zinc ions both inhibit guanylate cyclase activity and abolish certain of prolac-

TABLE IV. EFFECT OF VARIOUS ZINC ION CONCENTRATIONS ON THE INSULIN STIMULATION OF RNA SYNTHESIS.[a]

$ZnCl_2$ concentration (M)	[^{3}H]Uridine incorporation into RNA (dpm/μg RNA)		
	Control	Insulin	P
0	152 ± 11	345 ± 19	<0.01
1×10^{-4}	178 ± 13	210 ± 15	N.S.
5×10^{-5}	158 ± 12	262 ± 29	<0.01
2.5×10^{-5}	157 ± 8	306 ± 15	<0.01
1×10^{-5}	164 ± 13	298 ± 19	<0.01

[a] Explants were cultured for 1 day in the absence of hormones. Insulin and/or zinc was then added to the culture medium and incubation was continued for 4 hr. [^{3}H]Uridine, 1 μCi/ml, was added to the medium 1 hr prior to termination of the incubations. Numbers represent the mean ± standard error of explants from seven flasks.

tin's actions in mammary tissues. It is also possible, however, that other actions of zinc ions may be responsible for its inhibition of prolactin's effects. It was also of interest that the action of insulin on RNA synthesis was abolished by incubation with zinc ions. It is thus possible that the actions of insulin in mammary tissues may be influenced by cyclic GMP.

Recently, Manku *et al.* (5) in Horrobin's laboratory demonstrated that zinc ions at 0.012 mM imitate the actions of prolactin on vascular reactivity in the rat mesenteric vascular bed. It is indeed interesting the zinc ions at 10^{-5} M have no actions in mammary tissues, but imitate the actions of prolactin in the mesenteric vascular bed.

Summary. Zinc ions, at concentrations which were shown to inhibit guanylate cyclase activity, abolished the actions of prolactin on RNA and casein synthesis in cultured mouse mammary gland explants. These observations are compatible with the hypothesis that certain of the actions of prolactin in the mammary gland may be mediated by cyclic GMP.

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;ytotoxicity: Relationship to Tumorigenicity of Human Cell Lines in Nude Mice (40706)

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idies on immunity in human cancer carried out in allogeneic systems. e involved the use of cultured cells or as a source of antigen. Culture , however, may modify the char- of the original tumor type to such hat the cultured cells may become itative of the original tumor and, tly, inferior or useless in assays of cted immunity. Although in a few r cells may be identified in culture s such as melanosomes or CEA, res are defined only by the tumor rigin.

genicity in the nude mouse is a iterion of malignancy. On the other c of tumorigenicity in the nude ght be due to the effects of natural y (NC) in the nude mouse. Some tumor cell lines might be more o natural cytotoxicity and, as a ce, non-tumor producing in the .

cific natural cytotoxicity has been ted *in vitro* with material of human of rodent origin. The biological e of this reactivity remains un- . various studies the natural cyto- as been ascribed to different cell). Blocking experiments indicate roportion of effector cells, free Fc- re involved in the manifestation of totoxicity (4). Natural cytotoxicity gainst syngeneic as well as allogeneic cells also has been demonstrated le mouse (6–8). For these reasons cotoxicity appears to be exerted also lymphocytes.

rpose of the present work was to in a nude mouse model, the rela- etween tumorigenicity of estab- nan cell lines of malignant origin and the natural cytotoxicity *in vitro* against the same cultured cells.

Materials and methods. Mice. Female 5- to 8-week-old nude mice (BALB/c/ABOM Fib-nu [1 − $(\frac{1}{2})^{10-12}$]) were used. The mice were bred and raised under specified pathogen-free (SPF) conditions. At the age of 3 weeks the animals were transferred to minimum disease conditions with an atmospheric humidity of 80% and a room temperature of 28°C.

Established cell lines. Melanoma lines: RPMI 7931, Mel-1 (originating from O'Toole), Hu 831, Hu 866, Hu 1130, Hu 1214. Sarcoma line: SAOS-2 (originating from J. Fogh). Bladder carcinoma line: Hu 456. Fibroblast lines: Hu 808 cut and Hu 666 cut, both derived from normal skin. All cell lines were mycoplasma-free. Culture conditions were as previously described (9).

Effector cells. Spleen cells for use in the microcytotoxicity assay were obtained from untreated animals belonging to the same litters as those used in tumorigenicity experiments. Spleen cell suspensions were washed twice in a medium consisting of equal parts of RPMI 1640 and medium 199 (Gibco Bio-Cult, Glasgow, Scotland) with 10% fetal calf serum, penicillin, and streptomycin (25 IU/ml and 25 μg/ml, respectively). After lysis of contaminating erythrocytes with NH_4Cl, the cell suspensions were washed twice as above.

Assay for natural cytotoxicity. The microcytotoxicity assay (MCA) (10) was performed as previously described in detail (11). Briefly, the target cells for MCA were harvested from confluent monolayers by trypsinization. The same batch of cells was used for MCA as well as the tumorigenicity assay as described below. Ten-microliter volumes of cell suspension were seeded into each well of the microplates (Falcon 3034) and incubated for 18 hr at 37°C in a humidified gas mixture containing 5% CO_2. The effector cells were added.

d by the Danish Cancer Society.

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Lymphocyte/target cell ratios (L/T) ranged from 60/1 to 8000/1. The microplates were incubated for a further 48-hr period. At the end of this period the plates were fixed and stained. The number remaining were counted under a microscope.

Evaluation of results. A cytotoxic index was calculated as follows: $CI = (1 - L/M) \times 100$, where L = mean number of residual target cells in 12 wells after incubation with spleen cells, and M = mean number of target cells in 12 wells after incubation with medium only.

Tumorigenicity. For each cell line, approximately 30 nude mice comparable with respect to sex and age were divided into three groups. Animals in group 1 received 10^5 cells in 0.1 ml phosphate-buffered saline, whereas animals in group 2 and group 3 received 5×10^5 and 2.5×10^6 cells, respectively. The cell suspensions were inoculated sc into the right side of the chest wall. The areas of the individual tumors were measured in two dimensions and the volume was calculated using the smallest diameter twice (volume = $0.5236 \times \text{diam}_A \times \text{diam}_b \times \text{diam}_b$) (12).

Indirect immunofluorescence test. Sera from tumor-bearing and non-tumor-bearing animals were examined for antibodies against the cultured cell lines used for inoculation. The tests were performed using FITC-conjugated goat anti-mouse IgG, A, M (Nordic Immunology).

Results. Five out of six melanoma lines, as well as the sarcoma line, were found to be tumor producing in nude mice, whereas one melanoma, one bladder carcinoma, and two fibroblast lines were non-tumor producing. The tumor-producing capacity showed no correlation to passage number.

Observation periods were limited by the rate of spontaneous mortality, but ranged from 3–7 weeks. The number of animals with progressive tumor growth depended upon the size of cell inoculum. At the dose level of 2.5×10^6 cells, takes ranging from 7/7 to 9/10 were registered. In animals receiving non-tumor-producing cell lines no palpable tumors were observed in any instance.

It appears from Fig. 1 that in the MCA, spleen cells derived from untreated mice exerted a stimulatory effect ($CI < 0$) upon the growth of target cells obtained from tumor-producing cell lines. This effect appeared especially pronounced at L/T ratios above 1000/1. However, with some cell lines the effect of the spleen cells upon cell growth was pronounced even at low L/T ratios (125/1–250/1). In contrast to these findings, the growth of target cells derived from the non-

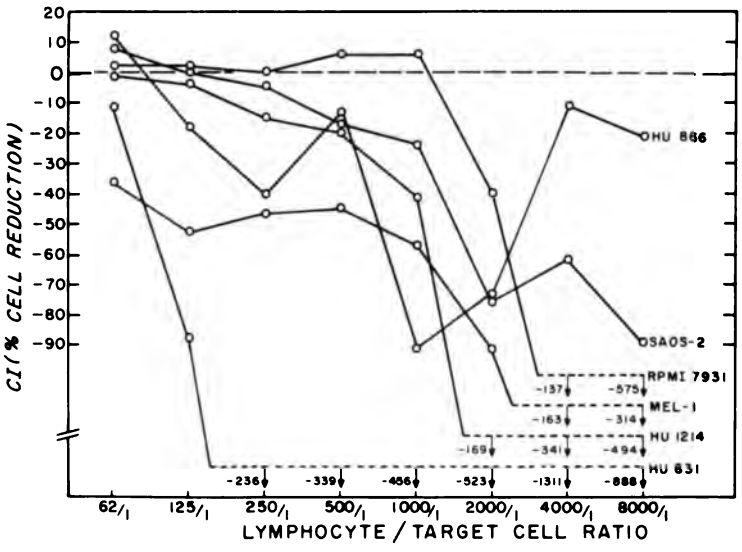


FIG. 1. Microcytotoxicity assay revealing a significant ($P \leq 0.001$) growth stimulation of tumor-producing cell lines by spleen cells derived from nontreated nude mice (L/T ≤ 2000/1). Melanomas: RPMI 7931, Mel-1, Hu 831, Hu 866, Hu 1214. Sarcoma: SAOS-2.

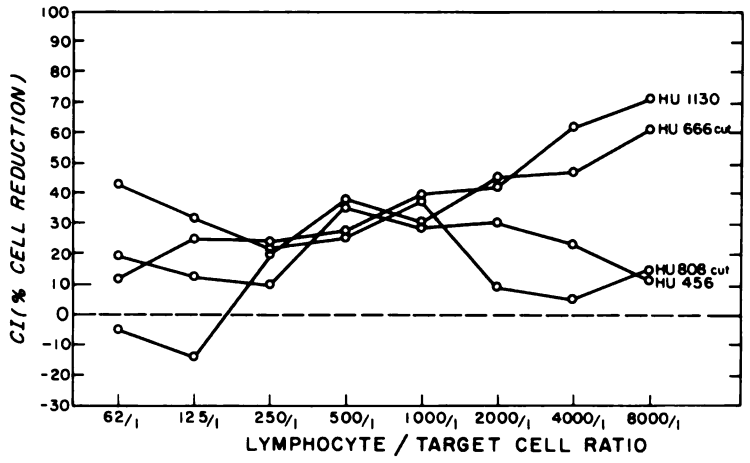
-producing cell lines was inhibited (CI
ıy spleen cells from untreated animals
:).
o case were humoral antibodies against
ɔculated cells demonstrated.
ussion. A growth-stimulatory effect of
ocytes has previously been reported
n vivo as well as *in vitro* experiments.
transplantation experiments (13) dem-
ted that immune lymphocytes, at a low
ocyte/target ratio, exerted a stimula-
fect upon tumor growth in a syngeneic
e model, whereas inhibition was ob-
at higher L/T ratios. Human lympho-
lerived from patients as well as control
ıs may also have a stimulatory potential
asured by the MCA (4, 11). In this
ı it has been observed that in some
he number of target cells is increased
ıpared to that of medium control dur-
: incubation ("feeder effect"). It should
ne in mind, however, that what is read
ɔtoxicity assays are the net results of
sis, detachment, and cell proliferation
er spontaneous or accelerated by a
r effect" due to the presence of lym-
tes. In contrast to the observations with
ırine models it is not known whether
ı lymphocytes under such experimental
ions are sensitized against the target. In
ɔf identical performance of the MCA,
a feeder effect or an inhibition was seen. The reason for this difference in pattern of reactivity is not known.

In the present work, using a xenogeneic model, a correlation was established between the tumorigenicity of human cell lines in nude mice and the *in vitro* stimulation exerted by nonsensitized spleen cells. The melanoma line RPMI 7931 has previously been used as the target in allogeneic MCA with lymphocytes derived from melanoma patients and control persons (4, 11). In these experiments, a few cases of stimulation were observed with patient lymphocytes as well as with control lymphocytes using L/T ratios ranging from 125/1 to 2000/1.

The finding that the *in vitro* growth rate of some cell lines (non-tumor producing) was inhibited, whereas the growth of other cell lines (tumor producing) was stimulated by the same effector cells, might indicate that tumorigenicity in the nude mouse may be determined by more than one factor.

It has generally been assumed that the immunological background which allows the support of xenogeneic tumor growth in nude mice is the lack of killer effector cells in the host. However, current experimental evidence may be interpreted to the effect that the nude mouse does possess cytotoxic effector cells different from killer T cells (6–8).

In the present work two out of four non-tumorigeneic cell lines were derived from



2. Microcytotoxicity assay disclosing growth inhibition of non-tumor-producing cell lines by spleen cells from untreated nude mice. Inhibition at the 19.63% was significant ($P \leq 0.05$). Inhibition exceeding nately 30% was significant at the $P < 0.001$ level. Fibroblast lines: Hu 666 cut, Hu 808 cut. Bladder na: Hu 456. Melanoma: Hu 1130.

malignant human tissues, i.e., melanoma and bladder carcinoma. The lack of tumor formation by these cells might possibly be explained as the result of selection of nonmalignant or stromal cells during cultivation. However, the lack of a tumor in the mouse does not definitely exclude malignancy, since the *in vitro* inhibition observed within such cells might reflect differential sensitivity among tumor-cell lines to the effect of natural cytotoxicity.

A stimulatory effect upon tumorigeneic cell lines was observed in the MCA with nonsensitized murine spleen cells. Provided that these *in vitro* observations reflect the existence of similar *in vivo* reactions, nonspecific cell-mediated reactions might support the early phase of tumor growth, whereas the capacity for immune-mediated inhibition of tumor growth may be acquired only at a later stage.

Summary. In the microcytotoxicity assay, a stimulatory effect exerted by nonsensitized spleen cells derived from nude mice was observed on the growth of tumorigeneic human tumor cells. In contrast, the growth of non-tumor-producing cell lines (malignant and nonmalignant) was inhibited by identical effector cells. Two out of four non-tumor-producing cell lines were of malignant origin. This lack of tumorigenicity, despite a derivation from tumor tissue, might be due to a selection of stromal cells during cultivation or might reflect a variation in the sensitivity to natural cytotoxicity independent of malignancy vs nonmalignancy. Consequently, lack of tumorigenicity does not exclude the possibility that the cells are malignant.

The technical assistance of Vibeke Ahrenkiel is gratefully acknowledged. I thank Lone Atke for careful secretarial assistance. This work was supported by Danish Medical Research Council Grants 512-5274 and 512-3547.

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INGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 471-479 (1979)

lic Adenosine 3′:5′-Monophosphate Mediation of the Effect of Dopamine on Renin Release by Renal Cortical Slices from Sodium-Deficient Rats: Modification by Dopaminergic and β-Adrenergic Receptor Blockade (40707)[1, 2]

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undant *in vivo* (1–4) and *in vitro* data have clearly determined that the sym- tic nervous system can directly influ- renin secretion by the juxtaglomerular of the mammalian kidney. This direct is primarily exerted by circulating cat- amines of adrenal medullary origin as as by norepinephrine released by renal athetic nerve terminals (10, 11). It is also that the direct stimulatory effect of nor- phrine on renin release is mediated by drenergic receptor mechanism (5–9, 12– which utilizes cyclic AMP as the intra- ar mediator (15, 16). Dopamine has also postulated as exerting a regulatory con- f renin secretion, either directly (17) or ctly (18–21), but the data are conflicting he mechanism involved in direct regu- remains unclear. In view of the impor- of dopamine as a central neurotrans- r (22, 23), and reports that dopamine is nt in renal tissue (24), further studies are y in order to examine a possible regu- role of this catecholamine on renin ion. The evaluation of a direct effect of nine on renin release in the live animal icult due to the interaction of humoral, dynamic, and neural factors, known to renin secretion. The utilization of *in* renal cortical preparations has effec- eliminated the contribution of humoral emodynamic influences, allowing the of stimuli which may directly affect release. Furthermore, *in vitro* studies in laboratory (25) and elsewhere (9, 15, 16) a rat renal cortical slice system, have n that the renin secretory responses to catecholamine stimulation are potentiated by dietary sodium deficiency, thus making an *in vitro* kidney slice system from sodium-deficient animals a more effective and sensitive tool for the evaluation of catecholamine effects on renin secretion.

In the present study, a hypersensitive renal cortical slice preparation from sodium-deficient rats has been employed to examine the possibility of a direct action of dopamine on renin release. Since dopamine-sensitive adenylate cyclase systems have been reported in the brain (26, 27) and kidney (28, 29), changes in renin secretion and tissue cyclic AMP content were simultaneously measured in this study, in an attempt to identify the type of mechanism mediating the renin release responses to added dopamine. Additionally, the effect of dopaminergic and β-adrenergic receptor blocking agents on renin release and tissue cyclic AMP content was examined, in an effort to characterize the type of receptor involved in these responses.

Materials and methods. Male Sprague–Dawley rats (Sprague–Dawley Co.) with initial weights of 210 ± 10 g were used in these studies. They were kept in temperature-controlled rooms (23 ± 2°C), two animals per cage, and had unlimited access to distilled, deionized water. The animals were fed a sodium-deficient chow diet (Teklad Mills Co.), which provided less than 0.02 meq of sodium per day, for 15 days. They were then sacrificed by decapitation and their kidneys rapidly excised, decapsulated, placed in Robinson's buffer medium (30), and gassed with 95% oxygen–5% carbon dioxide for 30 sec. Slices of renal cortex approximately 0.3 mm thick were subsequently prepared using a Stadie–Riggs microtome (A. Thomas Co.). Each cortical slice was further divided into various portions according to the number of treatments, and randomly assigned to separate incubating vessels containing 2.5 ml of

pported in part by Loyola University Research 333-39-226.

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whom requests for reprints should be sent.

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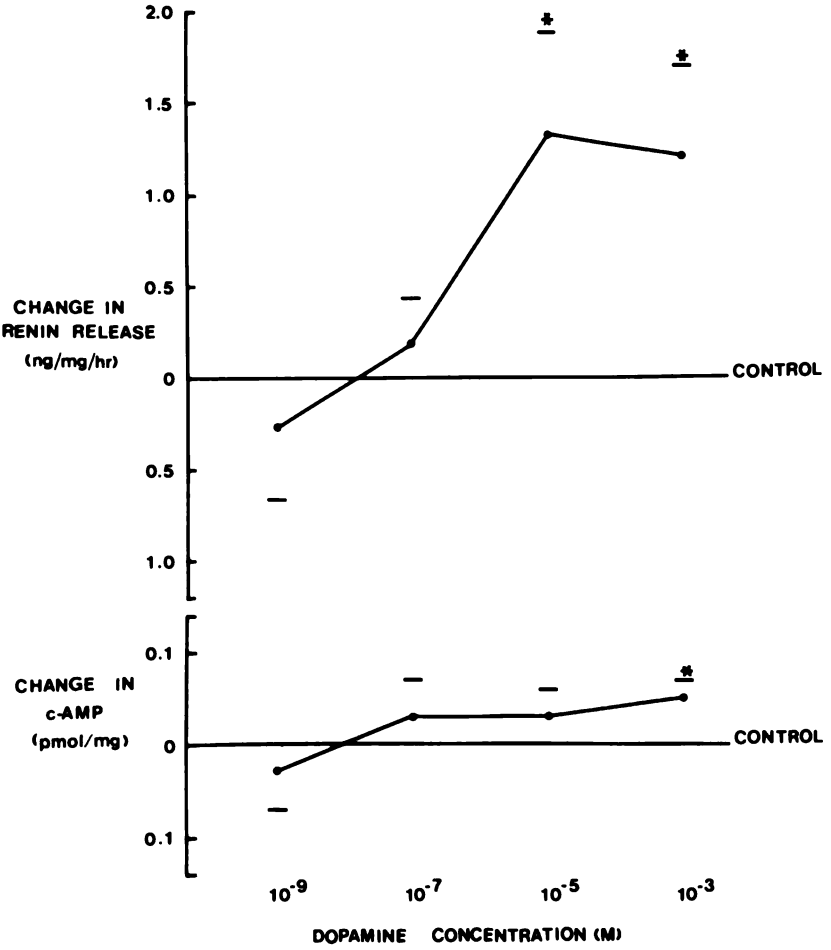
Robinson's buffer medium. This procedure was repeated using slices from other areas of the renal cortex, until each vessel contained 50 ± 20 mg of renal cortical tissue. The tissue distribution ensured a homogeneous cell population in the incubating vessels, and it allowed the simultaneous evaluation and comparison of various tissue treatments having a common control sample. Thus, one of each group of incubating vessels served as an untreated control. The cortical tissue was then preincubated for 15 min at 37°C in a shaking Dubnoff metabolic incubator (Precision Scientific), in an atmosphere saturated with 95% O_2–5% CO_2. The preincubated slices were subsequently transferred to vessels containing fresh Robinson's buffer at 37°C, and incubated for 1 hr under conditions identical to those of the preincubation period.

Dopamine (Sigma) solutions, prepared in 0.1% ascorbic acid (Sigma) to prevent oxidation, were added to the slice preparation before the 1-hr incubation period. The dopamine-receptor blocking agent pimozide (Janssen Pharmaceutical), similarly prepared in 1.2% tartaric acid (Sigma) to prevent oxidation, and the β-adrenergic receptor blocker propranolol (Sigma), prepared in Robinson's buffer, were in turn added to the tissue preparation before both the preincubation and incubation periods. In some studies, the dopamine-β-hydroxylase enzyme inhibitor FLA-63 (Regis Chemical Co.), prepared in 0.1% ascorbic acid, was added to the slice system before both the preincubation and incubation periods in order to prevent conversion of dopamine to norepinephrine in the slice tissue (31). Following incubation, the supernatant medium was collected and stored at −20°C until assayed for renin concentration. In turn, the tissue slices were rapidly frozen on dry ice, homogenated in 1 ml of 8% trichloroacetic acid, and extracted with water-saturated ether. The ether phase was then discarded while the cyclic AMP-containing water phase was lyophilized. Cyclic AMP content in the lyophilized samples was determined by a modification of the protein binding assay of Gilman (32), and the results were expressed as picomoles cyclic AMP/milligram wet tissue. Renin concentration in the incubated supernatant medium was measured by radioimmunoassay of angiotensin I (33), and the values were expressed as nanograms angiotensin I generated/milligram wet tissue/hour. The data were evaluated for statistical significance by modified paired and unpaired *t* tests (34).

Results. In an initial study, the effect of various dopamine concentrations on renin release and cyclic AMP content in renal cortical slices from sodium-deficient rats was examined. At the highest dose tested (10^{-3} *M*), dopamine significantly stimulated both renin release and tissue cyclic AMP content, but a lower dopamine dose (10^{-5} *M*) was only effective in significantly increasing renin release (Fig. 1). Maximal stimulation of renin secretion was observed at 10^{-5} *M* dopamine since a higher 10^{-3} *M* dose did not statistically potentiate the renin secretory rate. Although dopamine concentrations of 10^{-9} and 10^{-7} *M* did not significantly affect renin secretion, a dose–response relationship was observed at a dopamine range of 10^{-9} to 10^{-5} *M* when the renin release responses to these doses were compared to each other.

In a subsequent study, the specific dopamine-receptor blocker pimozide (10^{-6} *M*) was added to the tissue slice preparation either alone or in conjunction with various dopamine concentrations (Fig. 2). In the presence of pimozide, two previously stimulatory dopamine doses (10^{-5} and 10^{-3} *M*) were no longer effective in stimulating either renin release or tissue cyclic AMP content, and in fact a marked inhibition of the latter parameter was now apparent. Pimozide added alone, however, significantly inhibited both resting renin secretion and the cyclic AMP content in the incubated slice tissue.

Additional studies were designed to evaluate the possibility that the stimulatory effect of dopamine on renin release and tissue cyclic AMP content we had observed (Fig. 1) may have been due to the conversion of dopamine to norepinephrine in our tissue preparation rather than to a direct action of dopamine itself. In these studies, we utilized a specific dopamine-β-hydroxylase enzyme inhibitor, FLA-63, which effectively prevented dopamine from being converted to norepinephrine (31). In the presence of FLA-63 (10^{-4} *M*), a 10^{-3} *M* dopamine concentration significantly stimulated both renin release and tissue cyclic AMP content (Fig. 3) in a manner similar to



IG. 1. Effect of various concentrations of dopamine on renin release and cyclic AMP content in renal cortical ; from sodium-deficient rats. The data represent the mean renin release change ± SE and the mean tissue cyclic ' content change ± SE, respectively, of 12 observations for each dopamine concentration. The mean control treated) rate of renin release was 5.43 ± 0.19 ng/mg/hr and the mean control cyclic AMP content was 0.42 ± pmole/mg wet tissue. Incubation time was 60 min. * Significantly different from control ($P < 0.05$).

which we had observed in experiments 'hich FLA-63 was not used (Fig. 3). FLA- ıdded alone did not affect resting renin ase or tissue cyclic AMP content in this ly or in any other in which it was used. o further evaluate the possibility of a amine-specific renal receptor mediating stimulatory effect of dopamine on renin etion, a previously stimulatory dopamine : (10^{-3} *M*) was added to the slice prepa- ɔn either alone or together with pimozide $^{-6}$ *M*), with FLA-63 being present at all :s. As opposed to the results obtained in ious experiments in which FLA-63 was utilized (Fig. 2), the stimulatory effect of amine on renin release, still significant in the presence of the enzyme inhibitor, was now dramatically potentiated rather than inhibited by the addition of pimozide (Fig. 4). The significantly negative changes in tissue cyclic AMP content in response to pimozide added either alone or together with dopamine remained unchanged, however, whether FLA-63 was present or not (Figs. 2 and 4). A similar dissociation of the renin secretory responses from those seen in tissue cyclic AMP content was observed in another study in which various pimozide concentrations were added to the slice tissue preparation either alone or in conjunction with 10^{-3} *M* dopamine in the presence of FLA-63 (Fig. 5).

To determine if an alternative type of re-

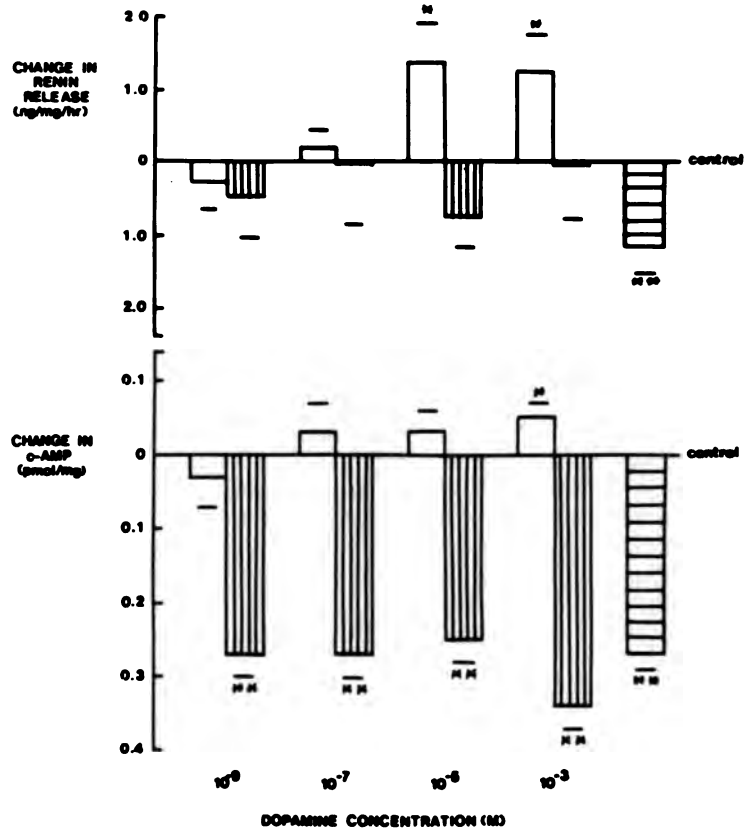


FIG. 2. Changes in renin release and tissue cyclic AMP content in response to various concentrations of dopamine, added either alone or together with 10^{-6} *M* pimozide to renal cortical slices from sodium-deficient rats. The data represent the mean renin release change ± SE and the mean tissue cyclic AMP content change ± SE, respectively, of 12 observations for each treatment. The mean control (nontreated) rate of renin release was 5.43 ± 0.19 ng/mg/hr and the mean control cyclic AMP content was 0.42 ± 0.02 pmole/mg wet tissue. Incubation time was 60 min. ▤, 10^{-6} *M* pimozide alone; □, dopamine alone; ▥, dopamine + 10^{-6} *M* pimozide. * Significantly different from control ($P < 0.05$). ** Significantly different from control ($P < 0.01$).

ceptor mechanism may be involved in mediating the stimulatory effect of dopamine on renin release and tissue cyclic AMP content seen in these studies, an additional study was undertaken. In this study, dopamine (10^{-3} *M*) was added to the slice system either alone or together with the β-adrenergic receptor blocker propranolol (10^{-4} *M*), in the presence of FLA-63 (Fig. 6). At the concentration used, propranolol effectively prevented the significant stimulatory effect of dopamine on renin release and tissue cyclic AMP content. Propranolol added alone did not affect renin release but it decreased tissue cyclic AMP content.

Discussion. Studies utilizing *in vitro* kidney preparations (5–9, 12–16) have provided evidence supporting the concept of a direct stimulatory effect of catecholamines on renin secretion from the juxtaglomerular cells of the kidney. In these preparations, the influence of blood pressure and macula densa flux changes which are known to influence renin release in the live animal is effectively eliminated, and although a number of cell types are still present the effect of sympathetic agonist and antagonist agents added to the tissue system is most likely a direct one on the renin-secreting cells.

The data reported in this study clearly indicate that dopamine is capable of directly stimulating renin release, which supports previous *in vitro* observations (17). Furthermore, these results show that the stimulation of renin secretion by dopamine is caused by this catecholamine itself rather than by its con-

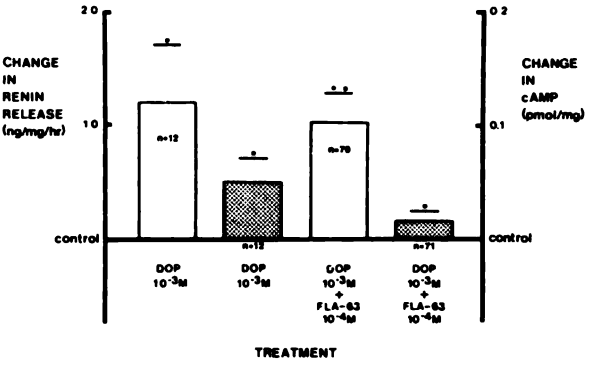
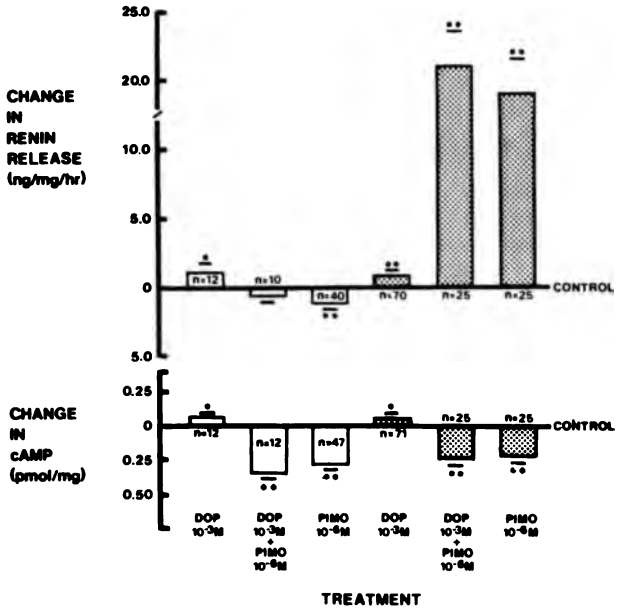


FIG. 3. Effect of dopamine (DOP) (10^{-3} *M*), added either alone or in conjunction with FLA-63, on renin release cyclic AMP content in renal cortical slices from sodium-deficient rats. The data represent the mean release ige ± SE and the mean tissue cyclic AMP content change ± SE, respectively, of 12 observations in experiments iout FLA-63 and of 70 and 71 observations, respectively, in experiments in which FLA-63 was used. The mean rol (nontreated) rate of renin release was 5.43 ± 0.19 ng/mg/hr in experiments without FLA-63 and 7.04 ± 0.32 xperiments with FLA-63. The mean control tissue cyclic AMP content was 0.42 ± 0.02 pmole/mg wet tissue in riments without FLA-63 and 0.32 ± 0.01 in experiments with FLA-63. Incubation time was 60 min. □, change nin release; ▨, change in cAMP; *n*, number of observations. * Significantly different from control ($P < 0.05$). ignificantly different from control ($P < 0.01$).



IG. 4. Changes in renin release and cyclic AMP content in response to 10^{-3} *M* dopamine (DOP) added alone gether with 10^{-6} *M* pimozide (PIMO) to renal cortical slices from sodium-deficient rats, in the presence or nce of FLA-63 in the incubating medium. The data represent the mean renin release change ± SE and the mean e cyclic AMP content change ± SE, respectively. The number of observations (*n*) per treatment is indicated. The n control (nontreated) rate of renin release was 5.43 ± 0.19 ng/mg/hr for DOP alone and DOP + PIMO ments and 5.23 ± 0.18 for PIMO added alone, in experiments without FLA-63; alternatively, it was 7.04 ± 0.32 ng/hr for DOP added alone and 7.7 ± 0.55 for DOP + PIMO and PIMO added alone, in experiments with -63. Similarly, the mean control tissue cyclic AMP content values were, respectively, 0.42 ± 0.02 and 0.40 ± 0.02 le/mg wet tissue in experiments without FLA-63, and 0.32 ± 0.01 and 0.31 ± 0.02 pmole/mg wet tissue in riments with FLA-63, for the same treatment distribution as for renin. Incubation time was 60 min. □, no FLA-3, with FLA-63 (10^{-4} *M*). * Significantly different from control ($P < 0.05$). ** Significantly different from control 0.02).

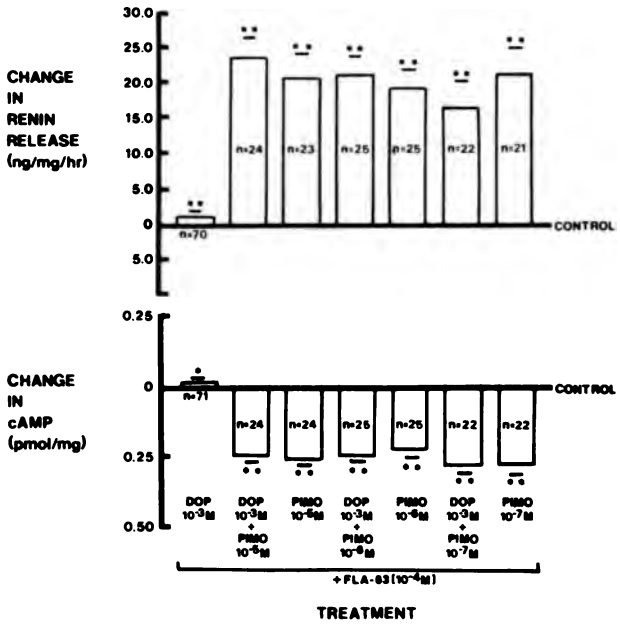


FIG. 5. Changes in renin release and tissue cyclic AMP content in response to 10^{-3} M dopamine (DOP) added alone or together with several pimozide (PIMO) concentrations to renal cortical slices from sodium-deficient rats. FLA-63 (10^{-4} *M*) was present in all instances. The data represent the mean renin release change ± SE and the mean tissue cyclic AMP content change ± SE, respectively. The number of observations (*n*) per treatment is indicated. The mean control (nontreated) rate of renin release was 7.04 ± 0.32 ng/mg/hr for DOP alone, 6.4 ± 0.53 for DOP + 10^{-5} *M* PIMO and 10^{-5} *M* PIMO alone, 7.7 ± 0.55 for DOP + 10^{-6} *M* PIMO and 10^{-6} *M* PIMO alone, and 4.4 ± 1.22 for DOP + 10^{-7} *M* PIMO and 10^{-7} *M* PIMO alone, respectively; similarly, the mean control tissue cyclic AMP content values were, respectively, 0.32 ± 0.01, 0.30 ± 0.02, 0.31 ± 0.02, and 0.34 ± 0.02 pmole/mg wet tissue, for the same treatment distribution as for renin. Incubation time was 60 min. * Significantly different from control ($P < 0.05$). ** Significantly different from control ($P < 0.01$).

version to norepinephrine in the slice tissue, since the significant stimulatory effect exerted by 10^{-3} *M* dopamine on renin release persisted in the presence of FLA-63, an effective dopamine-β-hydroxylase enzyme inhibitor (31).

The concept of a specific renal dopaminergic receptor mediating the effect of dopamine on renin secretion has been previously proposed (35, 36), although these data are inconclusive. Our results do not presently support the view that the stimulation of renin release and tissue cyclic AMP content seen in the presence of dopamine is mediated by a dopamine-specific receptor mechanism, since the meaning of our findings using the postulated dopamine-receptor blocker pimozide is unclear to us at this time. In our studies, three different pimozide concentrations added either alone or in conjunction with 10^{-3} *M* dopamine produced quite similar effects on either renin release or tissue cyclic AMP content. The tissue content of this nucleotide appeared markedly inhibited by pimozide in the presence or absence of FLA-63, but the presence of the enzyme inhibitor caused a dramatic stimulation of renin release by pimozide as opposed to a previously significant inhibition caused by this agent when FLA-63 was not added to the tissue preparation. We do not have a reasonable explanation for the significant dissociation of the renin secretory and tissue cyclic AMP content responses to added pimozide when FLA-63 was present in the incubate, but some studies (20) have questioned the specificity of pimozide as a dopamine-receptor blocker, by demonstrating its inability to prevent the stimulation of renin release by the dopamine-receptor agonist apomorphine in the dog. Thus, the observed effects may be due to a generalized action of pimozide independent of its association with a dopamine-specific membrane receptor. Alternatively, they may reflect a blockade by

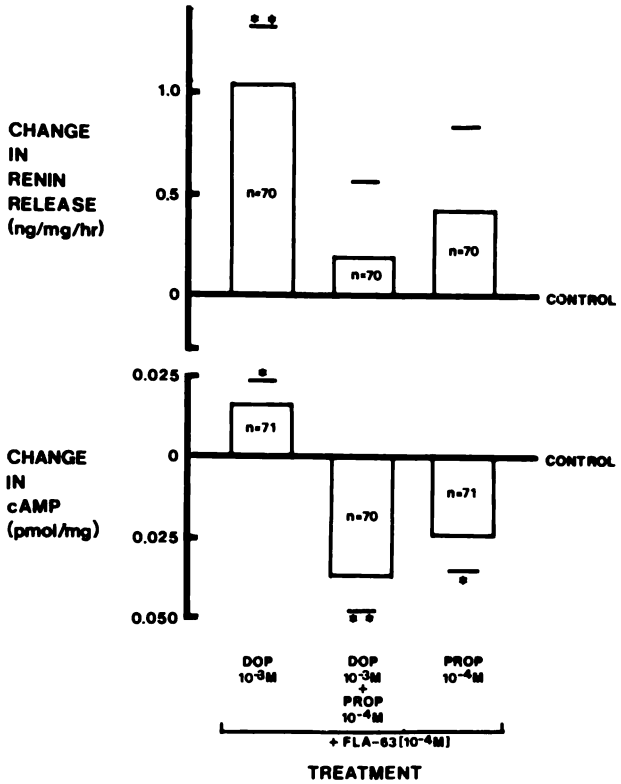


FIG. 6. Changes in renin release and tissue cyclic AMP content in response to 10^{-3} *M* dopamine (DOP) added alone or together with 10^{-4} *M* propranolol (PROP) to renal cortical slices from sodium-deficient rats. FLA-63 (10^{-4} *M*) was present in all instances. The data represent the mean renin release change ± SE and the mean tissue cyclic AMP content change ± SE, respectively. The number of observations (*n*) per treatment is indicated. The mean control (nontreated) rate of renin release was 7.04 ± 0.32 ng/mg/hr and the mean control tissue cyclic AMP content was 0.32 ± 0.01 pmole/mg wet tissue. Incubation time was 60 min. * Significantly different from control ($P < 0.05$). ** Significantly different from control ($P < 0.01$).

pimozide of dopamine-induced intracellular cyclic AMP content changes in cortical cells other than the juxtaglomerular cells, while dopamine may be simultaneously modulating both renin release and cyclic AMP content by a propranolol-blockable mechanism. Regardless, it is obvious that further *in vitro* studies utilizing additional dopamine-receptor agonist and antagonist agents, phosphodiesterase inhibitors, additional pimozide concentrations to find a dose which by itself does not affect renin release and cAMP content, and the measurement of intracellular renin changes are needed before the possibility of the existence of a dopamine-specific renal receptor modulating renin release can be properly evaluated. These studies are currently being conducted in our laboratory.

It has been proposed (22, 37) that structurally, dopamine has the capability of binding to various types of membrane receptors. Our data support this view by clearly showing that the simultaneous stimulation of renin release and tissue cyclic AMP content by 10^{-3} *M* dopamine was effectively prevented by the β-adrenergic receptor blocker propranolol. These results agree with previous observations (17), and they additionally suggest that the stimulatory effect of dopamine on renin secretion is at least partially mediated by a β-adrenergic receptor mechanism involving intracellular cyclic AMP changes.

Summary. The effect of dopamine (DOP), dopamine-receptor antagonist, and β-adrenergic receptor antagonist agents on simultaneously measured renin release (RR) and

cyclic adenosine 3':5'-monophosphate (cAMP) content of renal cortical slices from sodium-deficient rats was studied *in vitro*. A DOP dose of 10^{-5} *M* significantly increased RR while a higher (10^{-3} *M*) dose significantly stimulated both RR and cAMP content in the slice preparation. Addition of the DOP-receptor blocker pimozide (PIMO) at 10^{-6} *M* effectively prevented stimulation of RR and tissue cAMP content by DOP. However, PIMO added alone also significantly decreased resting RR and tissue cAMP content levels. In subsequent experiments, the DOP-β-hydroxylase (DBH) enzyme inhibitor FLA-63 (10^{-4} *M*) was used in conjunction with the other agents to prevent conversion of DOP to norepinephrine (NE) in the tissue preparation. In the presence of FLA-63, 10^{-3} *M* DOP again significantly stimulated both RR and cAMP content as previously seen without FLA-63. Conversely, a previously inhibitory effect on RR was converted to significant stimulation by several PIMO doses in the presence of the DBH enzyme inhibitor. Tissue cAMP content appeared significantly inhibited by all PIMO doses added alone or together with 10^{-3} *M* DOP whether FLA-63 was present or not. FLA-63 was ineffective by itself. A 10^{-4} *M* dose of the β-adrenergic receptor blocker DL-propranolol (PROP), added together with 10^{-3} *M* DOP and FLA-63, significantly prevented the stimulatory effect of DOP on RR and tissue cAMP content. These data indicate that (i) DOP itself can directly stimulate RR from the renal juxtaglomerular cells, (ii) a direct stimulatory effect of DOP on RR is at least partially mediated by a β-adrenergic receptor mechanism involving intracellular cAMP changes, and (iii) further studies are needed before the possibility of the existence of dopamine-specific renal receptors mediating the action of DOP on RR can be properly evaluated.

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Zinc in Erythrocyte Ghosts (40708)

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In our previous paper we demonstrated that a dietary supplementation of a high dose of zinc to rats renders red blood cells more resistant to osmotic hemolysis while an addition of Zn^{2+} to washed erythrocytes does not affect their osmotic fragility (1). On the other hand, erythrocytes from zinc-deficient rats which have low plasma zinc are more fragile under osmotic stress than those of controls (2). Bettger *et al.* (2) also showed that erythrocyte membranes are stabilized by zinc, while copper ions have a labilizing effect. Avigad and Bernheimer (3) demonstrated that hemolysis induced by bacterial toxin or other lytic agents is inhibited by Zn^{2+}. Montgomery *et al.* (4) showed similar zinc stabilizing effects using complement-mediated hemolysis.

Zinc was found to stabilize the integrity of lysosomes in *in vitro* experiments (5). As this effect was also produced by zinc–8-hydroxyquinoline unsaturated complexes (1:1, 1:2) which are impermeable to the membrane, we assumed that the effect of zinc is confined to the surface of the membrane (6). There are several possibilities to explain the stabilizing effect of zinc. Supplemental zinc may interact with intrinsic as well as extrinsic macromolecules of the membrane structure, thus contributing to their integrity (7). Yamamoto and Takahashi (8) found that Zn^{2+} suppressed complement-induced lysis of erythrocytes by acting directly on the erythrocyte membrane. In fact, zinc, as well as other inorganic ions, may be an integral part of various membranes, performing the same function as has been shown for calcium. As far as the effect on mechanical properties of membranes is concerned, a hardening effect of Ca ions on cytoplasmic surfaces or model membranes has been repeatedly demonstrated. Recent evidence suggests that zinc also increases the stability of various membranes. Zinc has long been used by biologists in the isolation of intact cell plasma branes from animal cells (9). Zinc an mium contribute to the assembly a integrity of brain microtubules (10). evidence of the membrane stabilizing of zinc and its presence in biomembr still lacking. The only reference ind that zinc is tightly bound to cell mem refers to the leaves of *Silene cucubatu* the membranes of which contain abo of the total zinc in the plant.

It is the aim of this study to pres evidence of the presence of zinc in eryth ghosts and to demonstrate some impli of the role of this metal in the structure membrane.

Materials and methods. Isolation blood cells. Whole blood from norma was collected in heparinized cont Fresh blood was centrifuged at 1000*g* a supernate and buffy coat were aspirate packed cells were then washed three with 15 vol of phosphate buffer (pH 7 mOsm) at 1000*g*.

Ghost preparation. Red cell mem were prepared by the procedure of D *al.* (12) by washing three times with 3(ice-cold hypotonic (20 mOsm) 5 m*M* phate buffer, pH 7.4. In order to s possible sequestration of metals fro ghost constituents by the complexing of phosphate, in some experiments we tuted phosphate with hypotonic barbi mOsm, 13.3 m*M*, pH 7.4) which do form a complex with zinc ions. Centrifu of the washed ghosts was done at 20,(an angle head for 40 min. A thoroug ration of the supernate at the surface pellet was done after each spin. This st repeated four to five times as needed u final pellet was white and the supe visibly free of hemoglobins.

Solubilization of ghost proteins. Gho tein solubilization was achieved with s

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ecyl sulfate (SDS), 1% in 0.01 *M* Tris
er, pH 8.5, containing 0.05 *M* NaCl. Ap-
imately 1 mg of ghost protein was dis-
ed in 100 μl of the above solution and
ibated at room temperature in the pres-
: of 0.05 m*M* sodium azide. The proteins
ie supernate were separated by spinning
5,000*g* and subjected to further analysis.
rotein separation. Protein separation was
ormed at room temperature in a Sepha-
G-200 (Pharmacia) column 30 cm long
1.5 cm in diameter, eluted at 8 ml/hr
i 0.01 *M* Tris buffer (pH 8.5) and 0.1%
). The proteins in the eluate were recorded
i DB-Beckman spectrophotometer at 280
For rechromatography the pooled sam-
were lyophilized without dialysis and run
er identical conditions (see Table I).
xtraction of ghost lipids. Total lipids of
ghosts were extracted by the procedure of
:h *et al.* (13) and determined by weighing
luminum weigh boats in a microbalance.
rder to obtain separation of major classes
iembrane lipids we used chromatography
silicic acid columns as described by
ser *et al.* (14). Aliquots of ghost lipids
matographed on silicic acid columns
: eluted first with chloroform, then with

BLE I. CONTENT OF ZINC IN MACROMOLECULAR COMPONENTS OF ERYTHROCYTE MEMBRANE.[a]

Fraction	Protein[b] (mg/fraction)	Zinc (μg/fraction)	Zn/protein (μg/mg)
1 I	0.35	0.29	0.83
2 II	0.64	1.00	1.56
3 III	0.41	0.17	0.41
4 IV	0.30	0.08	0.28
5 I-1	0.16	0.13	0.81
6 I-2	0.12	0.10	0.83
7 I-3	0.07	0.06	0.83
8 II-1	0.30	0.47	1.70
9 II-2	0.19	0.30	1.58
0 II-3	0.15	0.23	1.53
1 III-1	0.20	0.08	0.40
2 III-2	0.21	0.09	0.43

he table presents data of one representative exper-
from a total of five similar fractionation studies.
rotein, 1.8 mg, was put on the column; 94% protein
ecovered. Proteins solubilized from the dog ghost
fractionated on a Sephadex G-200 column. Four
ons (I–IV) were obtained. Fractions I, II, and III
rechromatographed and eight additional fractions
obtained.

methanol, and finally with acetone. In individual fractions the content of cholesterol, phosphorus, zinc, and copper was determined.

Assays. Protein was measured by the method of Lowry *et al.* (15). Phospholipids were determined by means of phosphate analysis according to Bartlett (16). The amount of phospholipids was calculated from total phosphorus content assuming a phospholipid/phosphorus ratio of 25. Cholesterol was measured by the procedure of Zak *et al.* (17). Total lipids were determined gravimetrically following the procedure of Folch *et al.* (14).

The content of zinc was measured by atomic absorption spectroscopy, using a Perkin-Elmer ASS, Model 305, with a Zn-selective lamp. The metal was determined either directly in solubilized samples or after nitric acid–hydrogen peroxide digestion of ghost samples.

Lipid peroxidation of the ghost was ascertained by measuring malondialdehyde and fluorescent products as described elsewhere (1).

Results. Zinc in the ghosts subjected to peroxidation. In our previous work we showed that in the presence of zinc ions the lipid peroxidation of biological structures was decreased (1). Although the mechanism of this effect of zinc is unknown, we speculated that one of the possible modes of Zn^{2+} action may be a direct interaction with either polyunsaturated fatty acids, mainly of phospholipid moiety, or reactive groups of membrane proteins, thus protecting these membrane components against peroxidative deterioration. An experiment was designed to test the function of zinc in relation to membrane constituents under the effect of peroxidative changes.

Hemoglobin-free ghosts, prepared from dog erythrocytes by the procedure of Dodge and co-workers (12), were incubated in the presence of ferrous ions and ascorbate, inducing lipid peroxidative changes in the membrane constituents (Fig. 1). The magnitude of lipid peroxidation was evidenced by measuring the formation of malondialdehyde and fluorescent product (top, Fig. 1). Parallel with the continuous increase in these parameters, both protein and phospholipids were

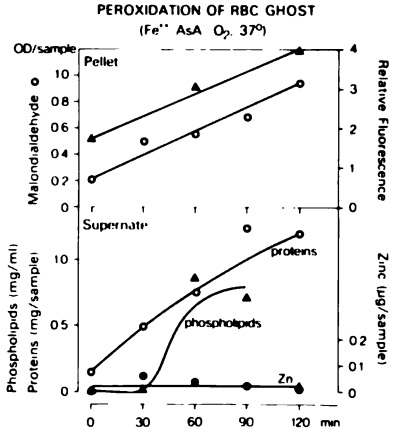

FIG. 1. Hemoglobin-free ghosts isolated from dog erythrocytes were incubated in 0.05 *M* Tris–0.15 *M* NaCl (pH 7.4) at 37°C in the presence of 200 μM L-ascorbic acid and ferrous sulfate. The medium was aerated for 15 sec with oxygen and incubated for given time periods under gentle shaking. Some biochemical indicators were determined in the pellet or in the supernate. The data of one representative experiment from a total of three similar experiments are presented. The variability for either parameter studied did not exceed 15% of the mean value.

released from the ghosts and leaked into the medium. No release of zinc from the ghost structure was observed during the 2 hr of incubation (bottom, Fig. 1).

Zinc and protein components of ghosts. Pure, hemoglobin-free ghosts were treated with 1% SDS in 0.01 *M* Tris buffer, pH 7.4, with the hope of solubilizing most of the membrane proteins (18). Chromatography on Sephadex G-200 column equilibrated with 0.1% SDS was further used to separate and rechromatograph the proteins.

A total of 12 fractions were obtained and analyzed for the content of protein and Zn (Table I). The results showed that zinc was present in every protein fraction obtained by this separation procedure. Proteins constituting peak 2 of the original Sephadex G-200 eluant contained three to five times more zinc per fraction than the other three original fractions.

Under rechromatography of the original four peaks of proteins (fractions 1–4, see Table I), peak 2 separated into three more fractions (8–10, see Table I), each of which contained a higher amount of zinc than the other proteins, irrespective of whether the data were expressed as zinc/fraction or zinc/milligram protein.

Effect of the method of ghost preparation on zinc content. Phosphate is known to form strong complexes with zinc, the logarithm of the complexity constant being 10.4 (19). As the most widely used method of preparing ghosts (12) uses hemolysis and washing with hypotonic phosphate buffer, we studied the possible sequestration of zinc by phosphate as compared to isomotic barbital buffer, which does not form complexes with zinc. The results (Table II) showed the erythrocyte ghost content of zinc to be three times lower in phosphate buffer. Another striking difference was that significantly less zinc linked to proteins in specimens prepared with phosphate buffer. It is interesting that in the course of extraction of erythrocyte ghosts by water, no release of zinc from the membrane was observed, while at the same time distinct amounts of calcium leaked out into the incubation medium. Still, the values reported here for the erythrocyte ghosts do not necessarily reflect the status of zinc in the membrane of intact erythrocytes.

Based on this experiment we learned that the method of preparation of ghosts is essential for the total content of zinc in the sample and that phosphate buffer displaced zinc preferentially bound to protein and minimally affected zinc bound to lipids.

Zinc content in lipid fractions of ghosts. We already indicated that in barbital-purified ghosts almost 69% of the zinc is linked to

TABLE II. EFFECT OF THE ISOLATION PROCEDURE ON THE GHOST ZINC CONTENT IN PURIFIED ERYTHROCYTES.[a]

	Barbital buffer	Phosphate buffer
Protein (mg/mg dry substrance)[b]	0.33	0.38
Lipid (mg/mg dry substance)[b]	0.67	0.58
Zinc (µg/mg dry substance)[b, c]	0.22 ±0.037	0.062 ±0.022
Zinc bound to proteins (% of total)	32.2	8.4
Chloroform–methanol (2:1) extractable (% of total)	68.8	91.6

[a] Refers to dog erythrocytes.
[b] Dry substance corrected for salts.
[c] Variability for zinc content is given by SD, based on determination of three to five independent ghost preparations.

ble II). The consecutive extractions from rabbit erythrocytes by chloıcetone, or methanol, as described aterials and Methods, were pernd the content of cholesterol and ipids was determined in each fracther with the content of zinc and able III). As expected, most of the ol was extracted into chloroform and ıe phospholipids were extracted into . The data show that phospholipidions contain more zinc per unit of lipid than the other two fractions. ontent in either fraction is similar to ent.

ion. As already indicated by Rey-) there is an increasing body of ntal data, which suggests that in at erythrocyte membrane system inortions are an important structural nt. The same author showed that all of the protein from human ghosts in aqueous media when inorganic re removed by chelating agents. The Burger *et al.* (20) also indicates that ring stability of bovine vs human rtes correlates with the actual conn of divalent cations in the memıe results of this study show that zinc ıonent of the membrane of red blood e findings that zinc is not released rthrocyte ghosts undergo peroxidarioration or partial solubilization er indicate that this metal possibly a certain framework of ghost strucinking to a component of the mem-e speculate that this rather insoluble l component is not extractable beits association with zinc. Based on culations we suggest that zinc contributes to the integrity of the erythrocyte membrane. More experimental evidence is needed to support this view.

There is a relative increase in binding of zinc to lipid phase. This linkage seems to be even more stable than zinc–protein linkage as phosphate buffer does not sequester the metal from the lipid moiety and releases zinc only from the protein. The finding that zinc remains within the ghost structure while oxidized lipids (measured as phospholipids) are released during the peroxidation cleavage of the fatty acids possibly indicates that those lipid components linked to zinc are not affected and disrupted by the oxidative process.

Recent findings on the correcting effect of zinc on sickle cell anemia brought several interesting observations, implicating zinc as a calcium antagonist in the red cell membrane (21, 22). Calcium incorporation into red blood cell ghosts was reduced by 50 to 80% in the presence of zinc in the medium (23). These studies indicate that metals in the ghosts exist in a state of dynamic equilibrium, reflecting the actual content of these metals in the whole blood. This would explain why dietary supplementation of zinc or zinc deficiency states affect the fragility of erythrocytes (1, 2). As shown by the study of Sastry *et al.* (24), the content of zinc in erythrocyte ghosts is negligible as compared to its content in the whole cell, where it is linked mostly to proteins. Our data show, however, that zinc is an integral part of the erythrocyte membrane.

Summary. Proteins and phospholipids but not zinc were released into the medium containing hemoglobin-free ghosts from dog erythrocytes incubated in peroxidation-inducing agents (ascorbic acid, Fe^{2+}, O_2). The

TABLE III. METAL CONTENT IN LIPID FRACTIONS FROM RBC GHOSTS.[a]

	Fraction[b] content	Cholesterol content (% of fraction)	Phospholipids[c] by weight (% of fraction)	Metal content (μg/g lipid)	
				Zn	Cu
ipid content (% of dry weight)	54.0	31 ± 4.5	51.2 ± 6.2	24.2 ± 4.5	64.0 ± 7.0
form fraction (% of total lipid)	44.0	60 ± 8.5	3.2 ± 0.4	72.0 ± 10.6	72.0 ± 9.6
ıe fraction (% of total lipid)	45.0	10 ± 0.8	10.9 ± 1.7	82.7 ± 11.2	110.3 ± 12.1
nol fraction (% of total lipid)	50.3	3 ± 0.2	78.5 ± 9.6	115.0 ± 9.7	128.0

to rabbit erythrocytes, variability given as $\bar{X} \pm$ SE.
to prercentage of total dry weight of RBC ghost.
ıolipid content calculated as $P_i \times 25$.

content of zinc in the pure ghost depends on the purification procedure; using phosphate buffer it amounted to 62 ± 22 μg/g protein, and using barbital buffer-extracted ghost it was three times higher.

Although most of the zinc was linked to the lipid phase of the membrane, there is a certain protein moiety of membrane proteins binding more zinc than the other protein fractions. Among various fractions of lipids, zinc is linked mainly to phospholipids and this linkage is not broken by phosphate buffer.

It is concluded that zinc is part of the erythrocyte ghost, linked to protein as well as lipid phase.

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ıbbit Cardiovascular Responses during PEEP before and after Vagotomy (40709)[1]

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ɛfractory hypoxemic patients with uni- ı pulmonary disease may improve arterial ;enation by use of positive end expiratory ;ure (PEEP). PEEP has been used to pand and stabilize atelectatic areas of the (1). However, PEEP may also produce ɛsired effects. Depending on the degree, P may be followed by an increase or ease in heart rate (2, 3), compromised iac output (4, 5), and reduced arterial d pressure (2, 6). Recently, it was dem- rated that the mechanical factor of a ced venous return was not the causative ıt for the fall in cardiac output and arte- ›ressure (5, 7), and increased attention to oreflex mechanisms during PEEP-in- ɔd lung distension was suggested (7).

echanoreceptors in the cardiopulmonary ›ns, which are sensitive to change in lung me or pressure, are considered to influ- vasomotor and cardiac autonomic activ- 2, 3, 6, 8). It has been suggested that these ptors are subserved primarily by vagally iated afferents in the dog (4, 6, 9) and the ıit (10–12). However, vagal cold block or ›tomy had little effect on the depression ırdiac output and only partially reversed lecreased arterial pressure resulting from tive pressure diffusion respiration in the aortic denervated, paralyzed rabbit (10). s, it is not certain that only vagally me- ɔd mechanoreceptors were involved.

dditionally, it is not clear how activation ɛardiopulmonary mechanoreceptors re- in modification of systemic baroreflexes. k *et al.* (4) recorded decreases in heart cardiac contractility, and vascular resist- during lung inflation in total cardiopul- ary-bypass canine preparations. During procedures they used rapid blood infu- in an attempt to control systemic blood ;ure and baroreflexes. However, their records indicate that a constant pressure was not maintained by this method. Mancia *et al.* (13) suggested a dominant cardiovascular role for the carotid baroreflexes over the cardiopulmonary mechanoreflexes when the two systems interact. However, their mechanoreceptor stimulus was an increase in blood volume and not lung inflation. Volume expansion is not a selective stimulus to cardiopulmonary receptors, because it has been shown that attenuation of cardiovascular reflex responses during infusion, while not altered by vagotomy, are abolished by sinoaortic denervation (14). Thus, the direct cardiovascular effects as well as the modulation of systemic baroreflexes by cardiopulmonary mechanoreceptors need further study.

The object of this work was to investigate the hypothesis that increases in lung expansion or pressure during PEEP effect changes in heart rate and blood pressure before and during isolation of the arterial baroreflexes. A second objective was to determine what portion of the cardiovascular responses to PEEP might be reflexly mediated by vagal afferents. To accomplish these objectives, heart rate and blood pressure responses were measured during stepwise increases in PEEP in the intact (no nerves cut), in the aortic denervated, and then in the vagotomized rabbit. Changes in mean right atrial pressure and intrathoracic pressure were used as indices of pulmonary responses to PEEP. In the aortic denervated rabbit, to identify modulatory influences on the systemic baroreflexes, responses to PEEP were measured before and during vascular isolation of the carotid sinus and concomitant electrical stimulation of the cranial end of the left aortic nerve.

Methods. Fifteen rabbits of either sex (2.5 ± 0.3 kg (SE)) were anesthetized with sodium pentobarbital via an ear vein (Diabutal; Diamond Laboratories, Inc., 30 mg/kg, iv). A light level of surgical anesthesia was maintained by supplemental administration through a cannulated femoral vein. The de-

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scending aorta (via femoral artery) and right atrium (via jugular vein) were also cannulated and connected to Statham P23Ac and P23BB strain gauges for recording of mean arterial (MAP) and right atrial blood pressures (MRAP), respectively. Heart rate (HR) was recorded via sternal needle electrodes connected to a Grass EKG/Tachograph coupler. Permanent records were made on a Grass 7 recorder. The trachea was cannulated via a tracheostomy and the animal's respiration was controlled. Blood gases and pH were determined from frequent small blood samples analyzed in an IL 213 (Instrument Laboratories, Inc., Lexington, Mass.). Through a midventral incision, the left and right aortic and vagus nerves were located in the cervical region, carefully isolated from surrounding tissue for about 1 cm, and looped with a loose thread for identification prior to sectioning. In five animals the intrathoracic pressure (IP) was measured via insertion of an air-filled, blunt-tip catheter through the diaphragm into the cavity of the left or right lung. The respective tissues, diaphragm to skin, were sutured airtight around the catheter and the pneumothorax was minimized. The catheter was then opened via a three-way connector to a Grass (PT5A) volumetric pressure transducer. Values for MRAP and mean IP are expressed relative to atmospheric pressure.

Carotid sinus isolation. A modified Moissejeff technique as developed by Schmidt *et al.* (15, 16), was used to permit reversible isolation of the carotid sinuses from the systemic circulation. Briefly, both internal carotids were tied beyond the sinus regions at approximately 0.5 cm from their points of origin. The external carotid artery was carefully isolated but remained patent. All other small vessels were ligated. Following heparinization (4 mg/kg iv, 150 units/mg, Hynson, Westcott, Dunning, Inc., Baltimore, Md.) the common carotid arteries were cannulated with an extracorporeal loop. This permitted perfusion of the sinus by the carotids (closed loop, CL), or pressure regulation of the sinuses, via an elevated pressure bottle, after occlusion of the external carotid arteries (open loop, OL). Under all conditions intrasinus pressure (ISP) was monitored by a pressure transducer (Statham P23Ac) in the extracorporeal system near the sinus. In every animal during the OL condition the ISP was held at equilibrium pressure (EP) for that animal. EP was that controlled ISP which reflexly resulted in an equal mean arterial pressure.

Control of respiration. Using a small animal respirator (Model 665, Harvard Apparatus), each rabbit's respiration was adjusted, to a low level of inspiratory positive pressure breathing (IPPB). Respiratory rate (40 to 60 cpm) and volume (15 ± 3 ml/kg) were adjusted to maintain arterial blood pH (>7.42) and blood gases ($pO_2 > 90$; $pCO_2 < 35$ mm Hg) and thereafter held constant. Exhalation was spontaneous through a tracheal T-tube connected to a low-resistance Silastic tube (0.6 cm i.d.). PEEP was regulated by submerging the opening of the exhalation tube in a calibrated column of water to 2.5, 5.0, or 7.5 cm H_2O.

Nerve stimulation. The rabbit was used for this study because it has easily identified aortic nerves which run separate from the vagi and subserve only baroreceptors (17). Additionally, reflex cardiovascular responses to electrical stimulation of the aortic nerve are well defined in this species (14, 17–20). Following data collection with all nerves intact, both right and left aortic nerves were cut and the left central end was placed on bipolar hook electrodes, platinum–irridium, for electrical stimulation (18, 19). Responses to left aortic nerve stimulation (LANS) were recorded with both carotid sinuses in the circulation (CL) and with both sinuses isolated (OL); pressures of the isolated sinuses were maintained constant at predetermined equilibrium pressure (EP). Responses to LANS were obtained under the above conditions both before and after vagotomy. Electrical stimulation was from a Grass S5 stimulator. Stimulus parameters were selected to maximize reflex cardiovascular responses as has been previously published (18–20). Briefly, stimulation bursts were composed of square wave impulses of 10 V and 0.3-msec duration. Stimulus frequency was 80 Hz with a burst duration of 25 to 30 sec.

Experimental procedures. All animals were instrumented as described above. A group of 10 animals were exposed to PEEP increases and LANS during CL and OL conditions. Eight of this group completed the experimen-

ies through aortic denervation and va-
ıy. One animal died following vagot-
and data from one animal were not
led in the group analysis because its
gas and pH analysis indicated a sus-
l metabolic acidosis. A second group of
nimals had additional instrumentation
ntrathoracic pressure measurements.
from this latter group were analyzed
ıtely from those of the first.

he first group, prior to any denervation, steady state was established with IPPB. PEEP was increased from 0.0 to 2.5, r 7.5 in H_2O. The level of PEEP was mized within and between procedures ch animal. Small arterial samples (0.3 ml) were drawn for gas and pH analysis h PEEP steady state. Both aortic nerves then cut and a second CL steady state stablished with IPPB unchanged. An al blood sample was drawn for analysis. EP increase was imposed and, following / state, LANS started and continued gh maximal heart and vascular response . LANS was terminated and cardiovas- parameters were allowed to return to mulus levels. Then OL responses were ıred. The ISP was held at EP and a of PEEP imposed followed by LANS. ›en OL conditions, for each PEEP level, ıimal was returned to the CL conditions. ving data collection in the aortic vated animal, at each PEEP level in the ıd OL condition with LANS, the vagi sectioned and the procedures repeated. : second group of five animals went gh essentially the same procedures but ut LANS. This second group was used ermine the mean right atrial, intratho- and transpulmonary pressure responses : and during IPPB with the various levels. Collection of data in this group was separate and followed that of the first.

Data analysis. All values were reported as means or mean differences ± the standard errors of the means (SEM). Statistical evaluation was made by use of the appropriate Student *t* test for paired or unpaired comparisons. Values of $P < 0.05$ were considered significant. Regression analysis employed programmed linear regression (Texas Instruments, Inc., Dallas, Tex.).

Results. In eight neurally intact anesthetized rabbits (Table I) increasing PEEP by 2.5, 5.0, or 7.5 cm H_2O resulted in a proportional increase in heart rate (HR) and mean right atrial pressure (MRAP) and a decrease in mean arterial pressure (MAP). Left aortic nerve stimulation (LANS) was not attempted in the intact animal. Following bilateral sectioning of the aortic nerves the significant HR change at high PEEP level was not found. However, MRAP and MAP responses were essentially unchanged as compared to the responses of the intact animal.

Cardiovascular and reflex responses to left aortic nerve stimulation (LANS) during PEEP increases. Figure 1 represents an example of the cardiovascular responses of one aortic denervated animal during an increase in PEEP to 5.0 cm H_2O (arrows on time scale) both before (Panel A) and during (Panel B) carotid sinus isolation. Note in panel A, with the loop closed (CL), that at the start of the PEEP increase, the initial falls in MAP and ISP are maximal and then have a partial recovery phase. These results are indicative of the buffering capacity of carotid baroreceptors. No recovery in MAP is evident during repetition of the procedure with ISP held constant in open loop (OL) condition (panel B). Heart rate (HR) during either CL or OL conditions was essentially unaltered by an increase in PEEP. With establishment of

TABLE I. CARDIOVASCULAR RESPONSES TO POSITIVE END EXPIRATORY PRESSURE (PEEP) IN EIGHT ANESTHETIZED RABBITS.[a]

Parameter	Control	Change following PEEP increase (cm H_2O) of		
		2.5	5.0	7.5
R (b/min)	290 ± 7.0	8.0 ± 0.7	12.6 ± 0.9*	18.1 ± 1.2*
AP (mm Hg)	92.0 ± 3.0	−6.6 ± 1.0*	−17.0 ± 3.1*	−25.9 ± 4.2*
RAP (mm Hg)	1.0 ± 0.4	1.2 ± 0.4	2.5 ± 0.7*	3.5 ± 0.9*

= heart rate; MAP = mean arterial pressure; MRAP = mean right atrial pressure; values are mean ± I

: 0.05 (paired analysis) for values significantly different from control.

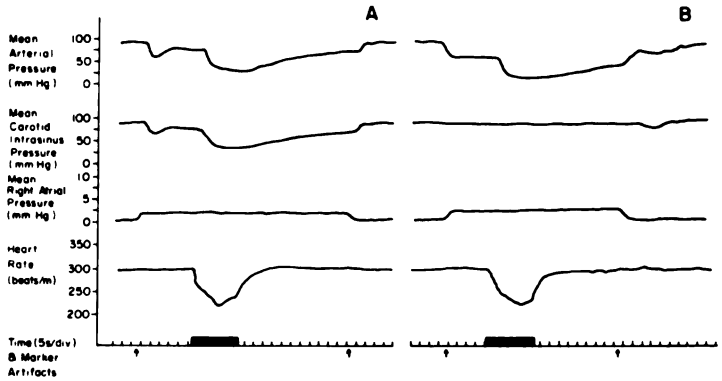

FIG. 1. Cardiovascular and reflex responses to left aortic nerve stimulation (LANS) during a 5-cm H_2O increase in positive end expiration pressure (PEEP) in one aortic denervated rabbit. Panel A, heart rate and vascular responses with the carotid sinuses able to detect systemic arterial pressure (closed loop (CL) condition). Panel B, heart rate and vascular responses during vascular isolation of the carotid sinuses (open loop (OL) conditions). Distance between arrows below the time line indicate the period of increased PEEP. Length of solid bars on time line indicates period of LANS.

steady state for HR and MAP during the PEEP increase, LANS was initiated (solid bars on time scale) and induced reflex falls in MAP and HR during both CL and OL conditions. The CL and OL maximal depressions in ΔMAP and ΔHR to LANS were recorded at each PEEP level, before and following vagotomy.

Heart rate responses to PEEP. The mean (± SEM) heart rates and ΔHR to LANS during CL and OL changes in PEEP for nine animals before (Panel A) and eight animals after (Panel B) vagotomy are presented in Fig. 2. Prior to aortic denervation HR responses (circled crosses) were progressively elevated during the CL state at each PEEP level (Fig. 2A, top; Table I). Following aortic denervation little change in HR responses (solid circles) occurred during CL increases in PEEP. However, HR responses (open circles) decreased slightly but significantly (10.0 ± 6.0 b/min) during the OL increase in PEEP of 7.5 cm H_2O when averages were compared to OL control (no PEEP) or the respective CL responses. No significant changes from control in CL or OL heart rates were observed after vagotomy at any PEEP level (Fig. 2B, top).

Reflex heart rate responses to LANS during PEEP. In aortic denervated animals the maximal fall (ΔHR) in OL heart rate responses (open circles) to LANS was significantly greater (average 17 b/min) as compared to

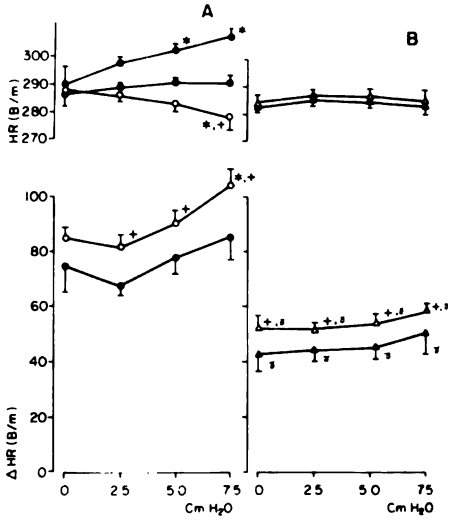

FIG. 2. Mean (± SEM) heart rate (HR) and peak reflex bradycardia (ΔHR) to left aortic nerve stimulation (LANS) during closed loop (CL) and open loop (OL) changes in PEEP for nine animals before (Panel A) and eight of the same after (Panel B) vagotomy. Circled crosses (⊕) indicate responses prior to aortic denervation; solid circles (●) indicate CL responses, and open circles (○) indicate OL responses of aortic denervated animals; solid triangles (▲) indicate CL responses, and open triangles (△) indicate OL responses following vagotomy; * indicates responses significantly ($P < 0.05$) different from control (no PEEP); + indicates OL responses significantly ($P < 0.05$) different from CL responses at the same PEEP level; and γ indicates responses significantly ($P < 0.05$) different when compared to respective pre-vagotomy responses.

of the respective CL responses (solid
:s) at all levels of increased PEEP (Fig.
ɔottom). When PEEP was raised to 7.5
I_2O, open loop ΔHR to LANS was sig-
ıntly increased (103 ± 10 b/min), as
ɔared to responses at 0.0 cm H_2O PEEP
ɛ 5 b/min). Following vagotomy both
and closed loop ΔHR to LANS were
ficantly reduced as compared to the re-
ive prevagotomy responses (Fig. 2B, bot-
. A significant difference (average 11 b/
persisted between OL (open triangles)
CL (solid triangles) responses when val-
vere compared before and during each
P increase, respectively. However, no sig-
ınt changes in OL ΔHR to LANS at any
P level were observed in the vagotomized
als.

*ɛan arterial blood pressure (MAP) re-
ɛes to PEEP.* MAP and reflex falls in
' (ΔMAP) to LANS for nine animals
'e and eight after vagotomy are summa-
in Fig. 3. The falls in CL steady state
' during PEEP in aortic denervated ani-
(Fig. 3A, top, solid circles) were not
ficantly different when values were com-
l with respective values for intact ani-
(circled crosses). In the aortic dener-
l animals, prior to PEEP, the control
' was 94.0 ± 5.0 mm Hg in the CL and
± 4.0 mm Hg in the OL. To remove
ılar influences from vagally mediated
opulmonary mechanoreceptors the vagi
sectioned. Vagotomy resulted in an in-
e in control MAP (Fig. 3B, top). Follow-
agotomy, CL MAP fell from 104.0 ± 4.0
Hg with each respective increase in
P and OL MAP fell from 97.0 ± 3.0 mm
espectively. CL MAP responses to PEEP
vels of 5.0 and 7.5 cm H_2O following
tomy were significantly different from
ıgotomy values. All OL MAP responses
:EP were likewise significantly different
vagotomy.

*flex MAP responses to LANS during
P.* Reflex blood pressure changes to
S are extremely sensitive to baroreflex
ɛensation from the carotid sinus barore-
ɪrs (20). Thus, to determine if PEEP
ts the reflex vascular responses during
S, responses were measured before and
ıg vascular isolation of the carotids. In
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ased fall in peak arterial blood pressure

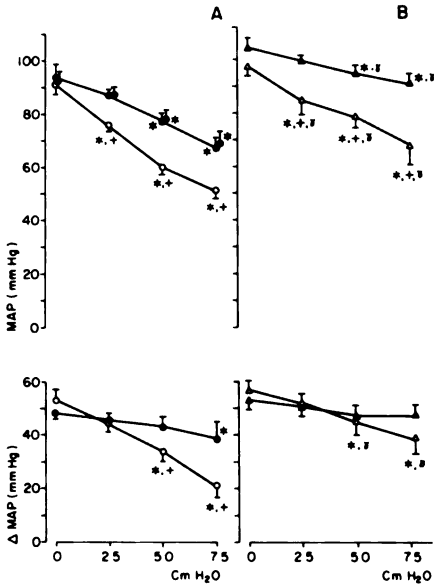

FIG. 3. Mean (± SEM) arterial pressure (MAP) and peak reflex fall in arterial pressure (ΔMAP) to LANS. Responses measured during CL and OL changes in PEEP in the same nine animals before (Panel A) and eight after (Panel B) vagotomy, as in Fig. 2. Symbols and abbreviations are the same as those in Fig. 2.

(ΔMAP) responses to LANS, during CL conditions, as each level of PEEP was increased (Fig. 3A, bottom, solid circles). CL control (0.0 cm H_2O PEEP) ΔMAP was 48.0 ± 2.0 mm Hg. In the OL condition, the reduction in MAP to LANS was even more evident as PEEP levels were raised (Fig. 3A, bottom, open circles). OL control ΔMAP was 53.0 ± 4.0 mm Hg.

Following vagotomy the CL and OL ΔMAP responses to LANS were greater with each increase in PEEP (Fig. 3B, bottom) as compared to the respective prevagotomy responses. CL control ΔMAP to LANS in the vagotomized animals was 52.0 ± 5.0 mm Hg, and OL control ΔMAP was 56.0 ± 3.0 mm Hg. However, again both CL and OL responses decreased with each increase in PEEP, respectively.

Mean right atrial pressure (MRAP) and intrathoracic pressure (IP) responses. Prior to vagotomy MRAP changes at each level of increased PEEP were not significantly different when respective responses of the intact (Table I) and aortic denervated animals were compared. In the aortic denervated animals with the carotids able to detect systemic blood

pressure (CL), MRAP increased from the control (no PEEP) value of 1.0 ± 0.6 mm Hg with each increase in PEEP. With the carotids isolated (OL) MRAP was 1.0 ± 0.6 mm Hg and also increased with each respective increase in PEEP. Following vagotomy MRAP again increased with added PEEP; however, responses were significantly ($P < 0.05$) reduced as compared to responses in the aortic denervated animal. Following vagotomy, with the carotids in the CL condition, control MRAP was 0.2 ± 0.9 mm Hg and 0.4 ± 0.9 mm Hg in the OL condition.

The significantly different MRAP responses following vagotomy suggest that transpulmonary pressures developed at each fixed PEEP level were altered when compared to respective prevagotomy values. To determine if this might be true in five rabbits the MRAP and mean intrathoracic pressure (IP) responses to PEEP were simultaneously determined during CL conditions for intact (no nerves cut), aortic denervated, and vagotomized animals (Figs. 4A–C). In the intact animals, prior to controlled ventilation, MRAP and IP were 0.7 ± 0.2 and −1.3 ± 0.1 mm Hg, respectively. With positive ventilation (O-IPPB) both MRAP (solid circles) and IP (crosses) increased. Then, as PEEP was increased both MRAP and mean IP responses increased in roughly parallel fashion. This relationship was not altered by aortic denervation (Fig. 4B) or vagotomy (Fig. 4C). However, while absolute MRAP and IP responses to PEEP were not significantly altered by aortic denervation, they were significantly ($P < 0.05$) reduced following vagotomy. For example, at 7.5 cm H_2O PEEP, MRAP and IP were 3.7 and 2.2 mm Hg in aortic denervated animals and 2.8 and 1.6 mm Hg after vagotomy, respectively. Transpulmonary pressure changes averaged 1.9 mm Hg, as calculated from the differences in IP responses to 0.0 and 7.5 cm H_2O PEEP both before and after vagotomy. However, the ranges of the transpulmonary pressure changes, calculated from either IP or MRAP changes, while still proportional to PEEP changes were shifted down following vagotomy.

Comparison of MAP and ΔMAP with MRAP responses during control and PEEP increases of 2.5, 5.0, and 7.5 cm H_2O. Following vagotomy with both CL and OL preparations, MAP, ΔMAP, and MRAP responses at each PEEP level were reduced when their respective values were compared to the prevagotomy response values, as noted earlier. It was considered that if vagal mediated pulmonary mechanoreceptors reflexly influenced vasomotor activity, then regression analysis of pre- and postvagotomy MAP (and

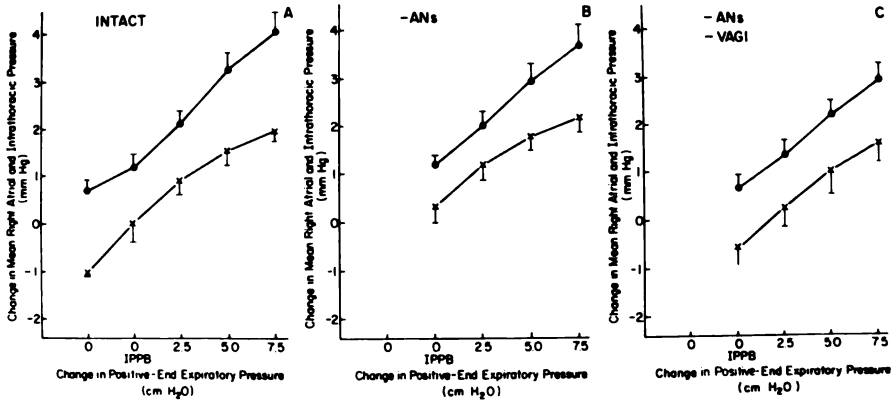

FIG. 4. Mean (± SEM) right atrial pressure (●) and intrathoracic pressure (X) responses before and during inspiratory positive pressure breathing (IPPB) and increasing positive end expiratory pressure (PEEP) of 0.0, 2.5, 5.0, and 7.5 cm H_2O in five rabbits. Panel A: Responses in the intact rabbits (no nerves cut). Values to the left of the IPPB zero were measured prior to cannulation of the trachea, and control of respiration. Panel B: Responses of the same animals following aortic denervation (−ANs). Panel C: Responses of the same animals following aortic denervation and vagotomy. Values were not significantly changed following aortic denervation (Panels A and B). All values following vagotomy were significantly ($P < 0.05$) reduced as compared (Student t test, paired observations) to prevagotomy values at the same level of IPPB and PEEP (Panels B and C). For further description, see text.

) with MRAP responses would indi- s by variation in regression slopes. A these responses and the regression ters involved are presented in Fig. 5 ble II, respectively. There was a sig- negative correlation between all CL \) and OL (Fig. 5B) MAP and MRAP es to the PEEP changes. There was a ant difference, however, between the nd open loop relationships. Similarly, AP to LANS responses were nega- orrelated to the MRAP responses dur- EP (Figs. 5C and D), while CL and ttionships were significantly different ompared. However, comparisons of postvagotomy regression relationships ot found significantly different within he CL or the OL condition.

ussion. Results of this study demon- hat both heart rate (HR) and mean pressure (MAP) are altered by an e in positive end expiratory pressure in the anesthetized rabbit. In the animal HR increased with each in- n PEEP (Fig. 2A, top). These results nilar to those observed by Hainsworth log (2). Following bilateral aortic de- on these HR increases were not ob- Since transpulmonary pressure has bserved to increase during PEEP both d in other studies (1, 5), it is possible rdioreflex activity from bronchopul- stretch receptors could be involved wever, under the present experimental ons the observed tachycardia can be ed only if afferent fibers from these receptors traveled in the aortic nerve. vidence for this possibility exists; in fact stimulation of the central end of the whole aortic nerve (17, 19, 20) or a few fibers (18, 21) resulted in a proportional reflex bradycardia. It is also possible that cardiac output alterations during PEEP (5, 8) may have produced the HR increase. However, following aortic denervation the pronounced tachycardia even at the higher PEEP levels was abolished. This would indicate that intrinsic

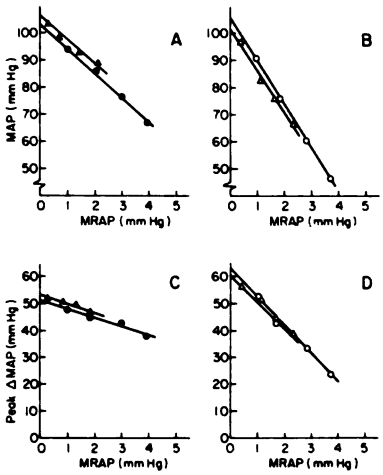

FIG. 5. Linear regression plots of MAP vs MRAP responses, and ΔMAP to LANS vs MRAP responses, during (left to right) 0.0, 2.5, 5.0, and 7.5 cm H_2O PEEP in nine rabbits before and eight after vagotomy. Panel A: MAP vs MRAP with carotid sinus in closed loop condition; (●) before and (▲) after vagotomy. Panel B: MAP vs MRAP with carotid sinus in open loop condition; (○) before and (△) after vagotomy. Panel C: ΔMAP to LANS vs MRAP with carotids in closed loop condition; (●) before and (▲) after vagotomy. Panel D: ΔMAP to LANS vs MRAP with carotids in open loop condition; (○) before and (△) after vagotomy. For further description, see text.

II. LINEAR REGRESSION ANALYSIS PARAMETERS OF MAP VS MRAP AND ΔMAP TO LANS VS MRAP, FOR NINE RABBITS BEFORE AND EIGHT AFTER VAGOTOMY, DURING 0.0–7.5 cm H_2O PEEP.[a]

ponse vs MRAP	Condition	Regression slope	Parameter intercept	Correlation (r)
IAP	CL prevagotomy (●)	−9.3	103.5	0.999
	CL postvagotomy (▲)	−7.9	105.5	0.973
	+OL prevagotomy (○)	−16.6	106.8	0.998
	+OL postvagotomy (△)	−15.7	101.6	0.966
IAP to LANS	CL prevagotomy (●)	−3.2	51.3	0.950
	CL postvagotomy (▲)	−3.0	53.1	0.886
	+OL prevagotomy (○)	−10.7	64.0	0.998
	+OL postvagotomy (△)	−8.9	60.2	0.985

onses and regression curves are plotted in Fig. 5. Symbols and abbreviations are the same as those in Figs. licates OL response relationship significantly ($P < 0.05$) different from respective CL response relationship. er description see text.

mechanisms of the heart were not a major factor in the response. The most likely possibility is that the transpulmonary pressure increase (Fig. 4) and systemic pressure decrease (Fig. 3) during PEEP cause a decrease in aortic transmural pressure. For example, prior to increased PEEP average aortic transmural pressure in the CL intact animals was calculated to be 92.4 mm Hg. With 7.5 cm H_2O PEEP this value fell to 64.5 mm Hg. This fall in aortic distension pressure would then be detected by the aortic sinus baroreceptors (22), with a resultant reflex increase in HR that is lost following aortic denervation.

In aortic denervated animals in which PEEP was increased 7.5 cm H_2O, and during control of the carotid sinus pressure (OL), HR was significantly reduced from control (0.0 PEEP) levels (Fig. 2A, top). This did not occur when the carotid baroreceptors were able to sense systemic blood pressure (CL) or following vagotomy. Also, during 7.5 cm H_2O PEEP and controlled carotid pressure, the ΔHR to LANS was significantly increased prior to vagotomy (Fig. 2, bottom). Previously, it has been indicated that tonic vagal restraint of the heart is minimal in the rabbit (14) and LANS effects a fall in HR by reflex suppression of cardiac sympathetics and stimulation of vagal efferents (14, 18). Thus, contrasting the results of pre- and postvagotomy HR and ΔHR to LANS responses during increased PEEP suggests two conclusions. First, the cardiac efferent vagal and/or sympathetic tone is altered during increased PEEP via vagally mediated cardiopulmonary mechanoreceptors, and second, the mechanical components of the PEEP levels used in this study has minimal effects on heart rate.

Following each PEEP increase MAP was depressed (Fig. 3A). Responses in the intact (circled crosses) and CL aortic denervated (solid circles) animals were essentially similar. These results demonstrate that aortic baroreflexes did not complement carotid baroreflexes in buffering the fall in systemic blood pressure during PEEP. These findings support the suggestion by Allison *et al.* (22) that aortic baroreflexes may have a "hypertensive role," as contrasted to the normotensive buffer functions of the carotid baroreflexes. With increased PEEP the MAP decreased during OL conditions (open circles) more than responses during CL conditions (solid circles). These data indicate the extent of buffering by carotid baroreflexes and suggest a modulatory rather than "dominant" role (13) for these arterial pressure receptors when they complement the cardiopulmonary mechanoreflexes during PEEP in the rabbit.

The peak reflex fall in MAP to LANS was reduced in the OL condition (open circles) as compared to responses in the CL conditions (solid circles) at PEEP above 2.5 cm H_2O (Fig. 3A, bottom). Earlier studies have indicated that electrical stimulation of the aortic nerve induced a reflex-mediated decrease in vasomotor tone (17, 19, 21). Thus, present results indicated that vasomotor tone was more reduced during PEEP when the carotid sinuses were vascularly isolated. Assuming a commonality of the vasomotor paths, as suggested by the work of Daly *et al.* (6), this decrease in vasomotor tone could, in part, be mediated by cardiopulmonary mechanoreflexes since carotid baroreflex influence was held constant during OL conditions.

Control (0.0 PEEP) CL MAP was increased following vagotomy (Table I, Fig. 3). These results are similar to those observed in the dog (2, 6, 9, 23) and the rabbit (10–12). With imposition of each PEEP increase the CL and OL fall in MAP (Fig. 3B, top) was less as compared to respective prevagotomy responses. In addition, the CL and OL ΔMAP responses to LANS (Fig. 3B, bottom) were significantly increased as compared to prevagotomy responses. These results indicate that vasomotor tone in both the CL and the OL condition is increased following vagotomy at all PEEP levels. However, since the OL fall in MAP and the decrease in OL ΔMAP to LANS, at increased PEEP levels, were still observed in the vagotomized animals, it is clear that the involvement of nonvagal factors is indicated. Daly *et al.* (6) suggested that the principal afferent fibers from the pulmonary receptors which interacted with baroreflex mechanisms during lung inflation ran in the vagosympathetic trunk with a lesser role attributed to fibers ascending through the stellate ganglia. Unfortunately, their open chest canine preparations did not allow quantification of transpulmonary pressure changes that could occur

; PEEP or following vagotomy (24, 25). nparison of MRAP and mean intratho- ressure (IP) responses, before and after my, indicated that proportional in- s in transpulmonary pressure occurred ach increase in PEEP (Figs. 4A–C). ving vagotomy both MRAP and mean re reduced for a given level of PEEP responses (Fig. 4C) were compared to otomy responses (Fig. 4B). These re- suggested that following vagotomy a e in the mechanical properties of the ccurs. With each increase in PEEP the tion of MRAP and IP responses was ained or slightly increased. Right atrial ural pressure in aortic denervated an- increased slightly from 0.8 mm Hg (0.0) to 1.5 mm Hg (7.5 PEEP) and from 1.2 mm Hg in the vagotomized animals, tively. These results indicated that transpulmonary pressure increased, net t atrial transmural pressure was main- even at 7.5 cm H_2O PEEP. These were similar to those found in the dog

rder to estimate the influence of vagally ted cardiopulmonary mechanorecep- n systemic blood pressure and the ca- and aortic baroreflexes during PEEP, closed and open loop MAP vs MRAP MAP (to LANS) vs MRAP were plot- r nine animals before and eight after my, and linear regression analysis was d (Fig. 5, Table II). Prior studies, in the (10–12) and dog (2–4, 6, 9, 13, 23), suggested a vagally mediated cardio- nary mechanoreflex depression of ar- blood pressure. However, these earlier s did not compare transpulmonary and ic pressure responses to PEEP pre- and gotomy. Results of the present study strate that as PEEP was increased, in- (MRAP and IP) of transpulmonary re increased while systemic arterial re decreased. Following vagotomy responses during PEEP were still pro- nal but reduced (Figs. 4B and C). It nsidered that the effect of vagally me- mechanoreflex activity on systemic pressure during PEEP should most be observed in the aortic denervated l when the carotid intrasinus pressure eld constant and the responses were ired before and after vagotomy. Figure 5B represents a plot of MRAP vs MAP responses to 0.0–7.5 cm H_2O PEEP before (open circles) and after (open triangles) vagotomy, with carotid intrasinus pressure held constant. Linear regression analysis did not indicate a significant difference between the relationships of these response curves. If vagally mediated cardiopulmonary mechanoreflexes had effected a depressor influence during PEEP, then the response curve following vagotomy (open triangles) should have had a more gradual slope than the prevagotomy response curve (open circles). Following a similar rationale, Fig. 5A indicates that carotid baroreflex activity was not modulated by vagally mediated mechanoreflexes during PEEP; Fig. 5D indicates that maximal aortic baroreflex depressor activity was not modified by vagally mediated mechanoreflexes during PEEP; and Fig. 5C suggests that vagotomy did not effect the carotid–aortic facilitory baroreflex potential. Thus, in the rabbit, vagally mediated cardiopulmonary mechanoreceptors have a minimal influence on systemic blood pressure or baroreflexes during PEEP. Vagotomy alters the mechanical properties of the rabbit's lung, and it is the mechanical and/or the nonvagally mediated cardiopulmonary receptor influences which affect systemic blood pressure or baroreflexes during PEEP.

Summary. Heart rate (HR), mean arterial pressure (MAP), and mean right atrial pressure (MRAP) were measured in 15 pentobarbital-anesthetized rabbits, with vascularly isolated carotid sinuses, during increases in positive end expiratory pressures (PEEP) of 2.5, 5.0, and 7.5 cm H_2O. Also, peak reflex bradycardia (ΔHR) and depression of arterial blood pressure (ΔMAP) to left aortic nerve stimulation (LANS) were recorded during the PEEP changes in aortic and subsequently vagal denervated animals. Responses were measured with the carotid sinus receptors detecting systemic pressure (closed loop (CL) condition) and with sinus region pressures held constant (open loop (OL) condition). Results demonstrated that both HR and MAP were altered by an increase in PEEP. Analysis of HR, and ΔHR to LANS responses during OL conditions, suggested the reflex involvement of cardiopulmonary afferent to cardiac efferent vagal fibers during PEEP changes. Vascular responses to increased

PEEP occurred in the presence or absence of arterial baroreceptors and, while reduced, were not abolished following vagotomy.

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ıtic Metabolism of Glycodehydrocholic Acid in the Dog and Rat (40710)[1, 2]

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ocholic acid (3,7,12-triketocholanic A, **Ia**, Fig. 1), a bile acid analog, is holeretic. It has been assumed that is due to the osmotic effect of DCA d into bile. Since DCA does not elles, if it or a derivative were exchanged, the compound would be alue in exploring the mechanisms taurocholate, a micelle-forming bile ances transport of various organic to bile (1–3).

ce for hepatic metabolism of DCA obtained, however, by several ins (4–11). In the most extensive loway and associates (7) found that) was totally metabolized in man, contained reduced metabolites **II**, V conjugated with taurine (Fig. 1). of products **II**, **III**, and **IV** was 20: ıolic acid (**IVa**) readily forms mimpounds **IIa** and **IIIa** are much less in micelle formation but they do ed micelles with **IVa**.

and Hanson (12) provided evidence odehydrocholic acid (GDCA, **Ib**) cted from metabolism. In isolated erfusion experiments, they reported CA was excreted unchanged into CA did not form micelles and did ıce the biliary excretion of the phosphatidylcholine. By contrast, ate (**IVc**) increased phosphatidylxcretion by 60% in the perfused rat

ıght to determine if GDCA is altered / injecting tritiated GDCA and exthe tritiated compounds excreted of the intact dog and rat. For comparison, DCA was also studied in several dogs.

Materials and methods. Chemicals. Sodium taurocholate, grade A, sodium glycodehydrocholate, grade A, and glycocholic acid (**IVb**) were purchased from Calbiochem, La Jolla, California; cholic acid (**IVa**), 5β-cholanic acid-3α,7α-diol-12-one (**IIIa**), 5β-cholanic acid-3α,12α-diol-7-one (**IIIa**), and 5β-cholanic acid-3α-ol-7,12-dione (**IIa**) from Steraloids, Inc., Winton, New Hampshire; dehydrocholic acid (**Ia**) and glycine ethyl ester hydrochloride from Sigma Chemical Company, St. Louis, Missouri; triethylamine from Matheson, Coleman and Bell, Norwood, Ohio; and EEDQ (*N*-ethoxycarbonyl-2-dihydroquinoline) from Aldrich Chemical Company, Milwaukee, Wisconsin. All were used as supplied except dehydrocholic acid which was recrystallized once from acetone. The steroid compounds all were at least 95% pure by thin-layer chromatography (tlc). Amberlite XAD-2 (Mallinckrodt Chemical Co., St. Louis, Mo.) was washed with methanol and stored under water.

[^{3}H]Glycodehydrocholic acid was obtained from New England Nuclear Corporation as a solution in ethanol. It was purified by tlc twice in solvent system 1 (see below) to give 170 μCi of 90% radiopure material. In solvent system 2 the impurities appeared as a broad, low-intensity band extending from the origin to the main peak, and in solvent system 1 some scans showed a small shoulder on the leading edge of the single peak. Evidently there were several small impurities, but no discrete peak corresponding to any of the compounds studied. The material weighed <5 mg, and it was stored as a solution in ethanol (12 μCi/ml). It had the same R_f as authentic glycodehydrocholic acid by tlc in our 2 solvent systems.

Glyco-5β-cholanic acid-3α,7α-diol-12-one (**IIIb**) was prepared by a procedure based on that of Tserng *et al.* (13) for synthesis of

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these studies were reported in abstract form terology **75**, 980 (1978).

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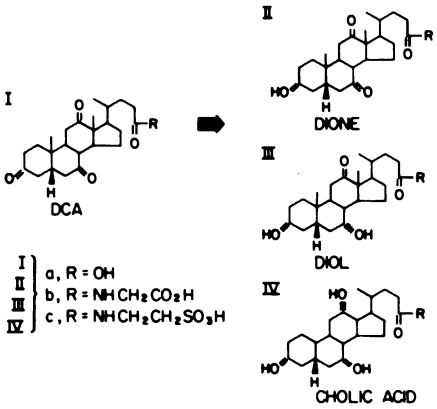

FIG. 1. Triketo bile acid metabolism.

glycine and taurine conjugates of bile acids. 5β-Cholanic acid-3α,7α-diol-12-one (**IIIa**, 80 mg, 0.197 mmole) with EEDQ (68.2 mg, 0.276 mmole) and glycine ethyl ester hydrochloride were converted into the corresponding glyco-diol-ethyl ester, 29.2 mg (61% yield). A second recrystallization gave 39 mg of crystals, pure by tlc. The ester (39 mg, 0.079 mmole) was saponified in hot ethanolic K_2CO_3 to give the glyco-diol **IIIb** as a white powder, 32 mg (89% yield). Thin-layer chromatography (system 2, see below) showed one spot (R_f 0.06).

Glyco-5β-cholanic acid-3α-ol-7,12-dione (**IIb**) was synthesized by a procedure similar to that used for preparation of glyco-diol **IIIb** above. 5β-Cholanic acid-3α-ol-7,12-dione (**IIa**, 100 mg, 0.247 mmole) was converted to the glyco-dione-ethyl ester (60 mg, 50% yield) obtained as a white solid from ethyl acetate–hexane. Thin-layer chromatography showed one spot (system 1, R_f 0.39; system 2, R_f 0.61). Saponification of this ethyl ester (60 mg, 0.122 mmole) gave glyco-dione **IIb** as a white powder (30 mg, 53% yield) pure by tlc (system 1, average R_f 0.32; system 2, average R_f 0.14).

Animal experiments. 1. Dog. Adult mongrel dogs weighing 15.5 to 18.6 kg were fasted for 12 hr prior to induction of anesthesia with intravenous pentobarbital (30 mg/kg). The cystic duct was ligated and common duct cannulated (PE-190) through a midline laparotomy incision. The renal pedicles were ligated in dogs 1 and 2. Body temperature was maintained at 37–38°C by heating pads under the dogs. Solutions were infused through PE-90 tubing in each jugular vein with a Bowman pump at rates of 1–1.5 ml/min. Infusion solutions of DCA–Na^+ were prepared by dissolving DCA and an equivalent amount of NaOH in pH 8 phosphate buffer (0.1 *M*) and diluting with normal saline; and GDCA–Na^+ solutions were prepared by dissolving the salt in normal saline.

Table I summarizes these experiments. In all dogs except dog 1, a constant taurocholate infusion was maintained through one jugular vein in order to replace bile acids lost in the interrupted enterohepatic circulation and thereby stabilize background bile acid excretion and bile flow. In each experiment after a period of stabilization of at least 30 min, a control bile sample was collected for 10 to 15 min before infusions or injections of GDCA or DCA were administered. Bile samples were collected in tared vials over 5- to 10-min periods.

2. Rat. Two adult Holtzman rats weighing 175 and 350 g were anesthetized with pentobarbital (30 mg/kg) injected intraperitoneally and the common bile duct was cannulated (PE-10). Bolus injections of GDCA–Na^+ in normal saline were made through a PE-50 cannula in the femoral vein. Injection volumes were ≤ 1 ml. Bile was collected for 5- to 10-min periods in tared vials before and after doses of GDCA. The experiments are summarized in Table I.

Analytical procedures. Bile volume was measured gravimetrically assuming a density of 1.0 g/ml. Bile salt concentrations in bile were determined by a 3α-hydroxysteroid dehydrogenase (Worthington Biochemical Corp.) method (14). DCA (**Ia**) and GDCA (**Ib**) did not react with the enzyme. Dione (**IIa**), glyco-dione (**IIb**), diol (**IIIa**), glyco-diol (**IIIb**), and cholic acid (**IVa**) as well as its glycine (**IVb**) and taurine (**IVc**) conjugates reacted similarly in the assay. Sodium and potassium concentrations in bile were measured by flame photometry (IL flame photometer, Model 143, Instrumentation Laboratory, Inc.), and chloride concentrations were determined by titration with a silver electrode (Buchler–Cotlove Chloridometer, Buchler Instruments, Inc.). Bicarbonate ion concentrations in bile were determined by the number of milliequivalents of HCl neutralized by an aliquot of the bile sample (15).

Thin-layer chromatography was per-

TABLE 1. SUMMARY OF GLYCODEHYDROCHOLATE AND DEHYDROCHOLATE STUDIES IN THE DOG AND RAT.

Animal No.	Body wt	Liver wt (g)	Compound administered	How administered	Dose (μmole/kg)	^{3}H dose (μCi)	Background taurocholate infusion (μmole/kg/min)
1. Dog studies							
1a	15.5 kg	475	[^{3}H]GDCA-Na$^+$	Bolus	Trace	24	—
b			[^{3}H]GDCA-Na$^+$ + GDCA-Na$^+$	Bolus	13.4	25	—
2a	18.6 kg	437	GDCA-Na$^+$	Bolus	49.6		0.16
b			DCA-Na$^+$	Bolus	49.6		0.16
3a	18.2 kg	625	GDCA-Na$^+$	Constant infusion	0.42/min		0.31
b					1.70/min		0.31
c					4.26/min		0.31
4a	18.6 kg	441	DCA-Na$^+$	Constant infusion	0.37/min		0.26
b					1.46/min		0.26
c					3.66/min		0.26
2. Rat studies							
1	350 g		[^{3}H]GDCA-Na$^+$	Bolus	Trace	12.3	—
2a	175 g		[^{3}H]GDCA-Na$^+$		50	0.63	—
b			+	Bolus	100	1.26	—
c			GDCA-Na$^+$		300	3.78	—

formed on 0.2-mm-thick silica gel plates without fluorescent indicator (EM reagents). Five-microliter samples of bile or solutions were applied and plates were developed with the following solvent systems: 1, isoamylacetate–propionic acid–*n*-propanol–H_2O, 40:30:20:10 (16); or 2, chloroform–methanol–acetic acid, 40:4:2 (17). Spots were visualized by spraying the plates with anisaldehyde reagent (18) and heating at 100°C for 10 min. Samples were dissolved in 10 ml of Biofluor scintillation cocktail (New England Nuclear) in 20-ml screw-cap glass vials and counted in a Packard liquid scintillation spectrometer, Model 3255.

Method A was used to identify tritiated metabolites in bile from animals injected with trace weights of [^{3}H]GDCA with high specific activity (dog 1a and rat 1). The bile sample containing the highest radioactivity (dog 1a, 3.1 ml, 10.9 μCi; rat 1, 0.7 ml, 8.4 μCi) was diluted to five times its original volume with water and acidified to pH 3.5 with 0.5 *N* HCl. This solution was slowly passed through a 5-g Amberlite XAD-2 column to absorb the bile salts. The column was washed with water until the eluant was neutral and then bile salts were eluted with absolute methanol. In separate studies [^{3}H]GDCA alone, or after addition to bile, was removed by the Amberlite column and subsequently was completely eluted with absolute alcohol. Evaporation of methanol under aspiration left a trace of residue which contained 87–100% of the starting radioactivity. Aliquots of the Amberlite-purified residue containing 0.5 to 2 μCi of tritium were streaked over 2 cm at the origin of tlc plates which were then developed in solvent systems 1 and 2. Standard compounds Ib to IVb were spotted on the plates for comparison. Spots were visualized with anisaldehyde reagent and plates were scanned with a Packard radiochromatogram scanner, Model 7201, to locate tritium activity. A second set of tlc's were run on samples to which authentic [^{3}H]GDCA was added (peak enhancement experiments). Peak ratios were measured from scans of plates run in solvent system 2. Attempts to apply enough unprocessed bile to the tlc plates to obtain an adequate scan were unsuccessful since severe tailing of bands resulted.

Method B was used to measure ratios of tritiated metabolites in bile from animals injected with a mixture of tritiated and cold GDCA (dog 1b and rat 2). Bile containing the highest tritium activity was applied at the origin of the tlc plate in four spots (5 μl each) and compounds **Ib** to **IVb** were spotted for comparison. Plates were developed in system 2 and spots visualized with the anisaldehyde reagent. Bands corresponding to the reference compounds were cut and leached with 1 ml methanol in 20-ml vials for 24 hr. Then 10 ml of scintillation cocktail was added and vials were counted.

GDCA metabolites in bile in dog 3 were estimated semiquantitatively as follows: A bile sample near the end of each of the three infusion periods was analyzed. A set of ten 1: 1 serial dilutions was prepared for each bile sample and for standard solutions of GDCA–Na^+ and glycocholic acid. Then 5 μl of each dilution was spotted on a tlc plate. Reference compounds **Ia** to **IVa** were also spotted for comparison. Plates were developed in system 2 and spots visualized with anisaldehyde reagent. The size and the intensity of the color of the spots of compounds excreted into bile were related visually to those of known concentrations of the standards. The limit of detection for GDCA and glycocholic acid was similar (0.2 nmole). Bile from each infusion 3a to 3c contained approximately equal amounts of glycocholic acid and GDCA.

Results. Injection of trace molar amounts of [^{3}H]GDCA into rat 1 resulted in excretion of 81% of the label in bile in 25 min. Similar injection of [^{3}H]GDCA into dog 1 (1a) led to recovery of 56% of the label in bile in 60 min. These bile samples were analyzed by tlc after isolation of bile salts by Amberlite XAD-2 column purification (see method A above). Thin-layer chromatography (Fig. 2) showed two peaks in system 1 and three peaks in system 2 but no unchanged GDCA was present in bile. Peak enhancement with authentic [^{3}H]GDCA in system 1 confirmed the absence of GDCA. Since the most likely metabolites were the reduced compounds **IIb**, **IIIb**, and **IVb** which are glycine conjugates of the compounds known to be formed from DCA (7), we synthesized glycine conjugates **IIb** and

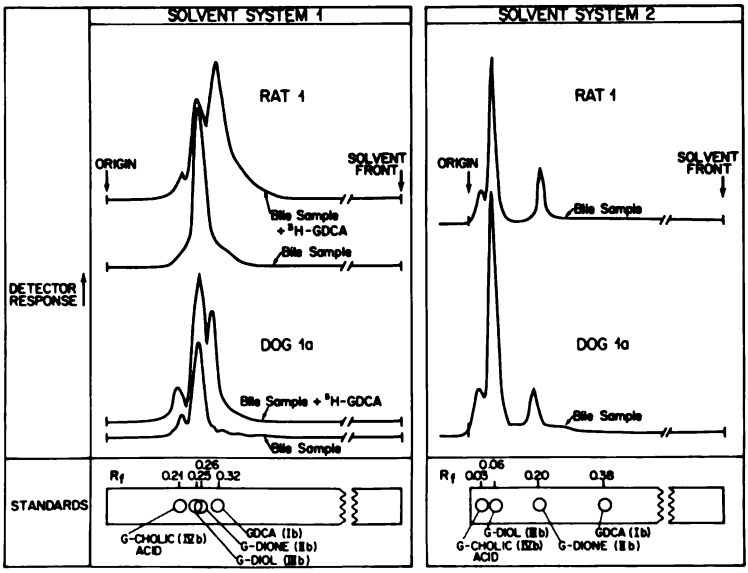

FIG. 2. Chromatograms of GDCA metabolites in bile. Radio-tlc of bile from rat 1 and dog 1a given [^{3}H]GDCA by vein in trace molar doses. Bile samples processed by Method A were run in tlc systems 1 (left panel) and 2 (right panel), respectively. Two radioactive peaks in bile were identified in system 1 and three peaks in system 2. Addition of [^{3}H]GDCA to bile resulted in the appearance of a new radioactive peak in system 1. The original two peaks in bile were not enhanced by [^{3}H]GDCA. System 2 provides better separation of the radioactive compounds excreted in bile. The three peaks in system 2 have mobilities identical with those of standard compounds **IIb** (G-dione), **IIIb** (G-diol), and **IVb** (G-cholic), respectively.

ɔm the commercially available bile
a and IIIa by the coupling procedure
ıg *et al.* ((13); see Materials and Meth-
ıe synthetic glyco-dione **IIb** and glyco-
b exhibited identical tlc mobilities to
f the two major metabolites in bile,
third metabolite corresponded to gly-
c acid (**IVb**) by tlc R_f value. System 2
d much better separation of **IIb** and
ın system 1, explaining why only two
lite peaks were found in system 1, but
eaks in system 2. Ratios of peak areas
cans of plates developed in solvent
2 gave the relative amounts of metab-
Table II). The ratios of reduced me-
s **IIb–IVb** in bile were similar in dog
rat 1.

bolites were not isolated and charac-
Structure assignments were made by
to the work of Soloway *et al.* (7) and
ty of tlc mobilities to those of authen-
pounds. Since glyco-diol **IIIa** and the
3,12-diol are not separated by our
systems and the corresponding gly-
ıjugates are probably not separated,
unable to distinguish these isomeric
ınds as metabolites. Ostrow and as-
(19) recently reported excretion of
-diol instead of the 3,7-diol in the bile
infused with taurodehydrocholate.

venous boluses of mixtures of tritiated
d GDCA were also injected into dog
nd rat 2. Bile from these animals was
d for metabolites by method B (see
These experiments showed the ap-

II. Relative Percentage of GDCA Metab-
in Bile Measured by tlc after Intravenous
istration of [^{3}H]GDCA in the Rat and

	Rat 1	Rat 2			Dog 1	
ent		a	b	c	a	b
of						
g)	Trace	50	100	300	Trace	13.4
	A	B	B	B	A	B
e						
	0	3	3.3	4.2	0	3
	15	59	47.7	62.1	9	48
	81	35	42.6	29.1	86	38
	4	3	6.4	4.6	5	11

lative percentage of metabolites determined by
can of Amberlite XAD-2 isolated compounds.
e percentage determined by elution and scin-
ounting of spots from direct tlc of bile.

pearance of small amounts of unchanged GDCA (**IIb**) in bile when the injected dose was 13 μmole/kg or greater (Table II). The relative proportion of glycocholic acid (**IVb**) in bile remained fairly constant regardless of dose, but that of glycodiol **IIIb** (or its 3,12 isomer) decreased while that of glyco-dione **IIb** increased with increasing administered doses of GDCA.

In dog 2 equal molar boluses of GDCA and DCA produced very similar choleretic effects (Fig. 3). The 3α-hydroxysteroid assay also showed similar excretion patterns. Thin-layer chromatography (solvent system 2) showed only small amounts of GDCA in bile from experiment 2a and a trace of DCA in bile from experiment 2b. Thus the excretion of 3α-hydroxymetabolites after injection of GDCA and DCA appeared similar. This is support for extensive metabolism of GDCA as has been reported for DCA (7) with the formation of 3α-hydroxymetabolites.

Constant infusion of GDCA (dog 3) and DCA (dog 4) into dogs also gave similar choleretic responses (Figs. 4 and 5). Bile samples were analyzed for 3α-hydroxysteroid compounds and for electrolytes for calculation of anion gap ($Na + K - Cl - HCO_3$). If appreciable unchanged GDCA or DCA, which are anions not measured by 3α-OH steroid assay, appeared in bile, there would be a discrepancy between 3α-hydroxysteroid excretion and anion gap excretion. Figures 4 and 5 show that the 3α-OH bile salt excretion approximates the organic anion excretion reflected by anion gap. This technique is consistent with extensive reductive metabolism of both GDCA and DCA to 3α-hydroxymetabolites. Thin-layer chromatography of bile samples from dog 4 showed only traces of DCA. The taurine-conjugated DCA metabolites in bile from dog 4 were not separated from intrinsic taurine-conjugated bile salts by our tlc systems. Bile from dog 3 showed spots corresponding to authentic **IIb**, **IIIb**, and **IVb** and a small definite spot for unchanged GDCA. The amount of GDCA in bile of dog 3 was estimated by a semiquantative dilution technique using tlc. This showed the amount of GDCA was small relative to glyco-diol **IIIb** and was approximately equal to the glycocholic acid (**IVb**) present in bile. Thus DCA was essentially completely reduced and con-

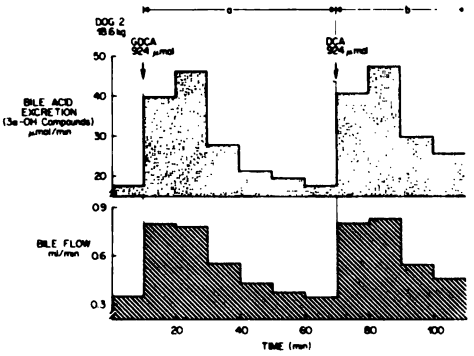

FIG. 3. Bile acid excretion and bile flow after administration of GDCA and DCA. Bolus injections of the respective compounds were administered in a dose of 924 μmole as indicated. Comparable amounts were excreted in bile over the ensuing periods (668 μmole, 72% of injected in 60 min for GDCA; 725 μmol, 79% of injected in 40 min for DCA).

jugated with taurine, whereas when GDCA was administered a small but definite amount of GDCA was excreted unchanged.

Discussion. Metabolism of GDCA was studied in dogs and rats given [^{3}H]GDCA and cold GDCA in various doses. Bile was examined by tlc for metabolites since GDCA- and glycine-conjugated metabolites are well separated by tlc from intrinsic taurine-conjugated bile salts in the dog and rat. The efficient separation of glycine conjugates in solvent system 2 allowed measurement of compound ratios directly by radio-tlc without tedious chemical conversions and possible alteration of compound ratios.

We found complete metabolism of GDCA at low doses and found 3 compounds which correspond to 3α-hydroxymetabolites **IIb**-**IVb** by tlc. We prepared glyco-dione **IIb** and

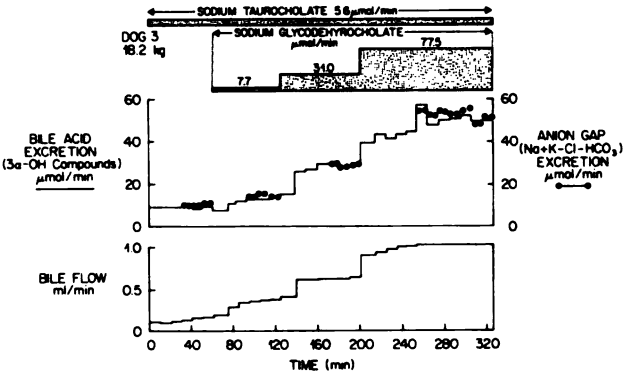

FIG. 4. Bile acid excretion and bile flow during infusion of glycodehydrocholate. Sodium glycodehydrocholate was infused at three separate constant infusion rates as indicated. Bile acid excretion based on measurements of 3α-OH compound excretion in bile and that based on measurements of anion gap excretion are virtually identical.

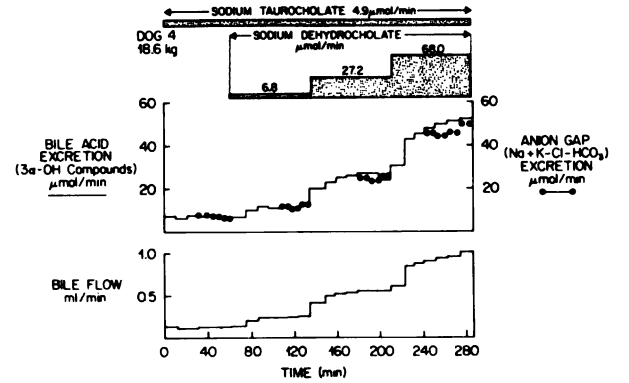

FIG. 5. Bile acid excretion and bile flow during infusion of dehydrocholate. The design of the experiment and the presentation of the data are similar to those of the experiment shown in Fig. 4.

)-diol **IIIb** by the coupling procedure of ng *et al.* (13) for tlc comparison. Our lts are analogous to those of Soloway *et* ') for metabolism of DCA. These authors venously infused ^{14}C-labeled DCA at doses (~10 μmole/kg) and metabolites ted in bile as taurine conjugates were njugated, esterified, and identified by tlc parison with authentic methyl esters of **IVa.** Reduction of keto groups in DCA ared to occur sequentially at C3, then and finally C12 positions. Random re- ion of DCA or GDCA could produce 26 ible hydroxymetabolites, and other hy- ylation products are also possible. How- , the unavailability of all these com- nds precluded our rigorously excluding 1 as possible metabolites. Metabolites **IIb IIIb** were not isolated from bile but there identical tlc mobility of the three metab- s of GDCA and compounds **IIb-IVb** in tlc solvent systems. We could not distin- n diol **IIIb** from the isomeric 3,12-diol by tlc systems. In a recent preliminary re- , Ostrow and associates demonstrated the 3,12 diol appears in bile of the rat wing administration of taurodehydro- ate (19). This further supports our con- on that **IIIb** may be the 3,7 or 3,12 er. However, the similarity of our prod- atios of GDCA metabolites with those of way *et al.* for DCA metabolism (20:70: **Ia:IIIa:IVa**) indicates the two compounds handled similarly on passage through the .

oses of GDCA above 13 μmole/kg in the and rat resulted in biliary excretion of ll amounts of unchanged GDCA (Table Increasing doses of GDCA resulted in ease in the relative amount of metabolite **IIIb** (or its 3,12 isomer) and proportion- ncrease in metabolite dione **IIb.** This may ct relatively less efficient dihydroxy for- on compared to monohydroxy formation ie load of GDCA is increased. The pro- ion of fully reduced metabolite **IVb** was ively constant at all infusion rates.

travenous GDCA and DCA produced lar choleretic effects whether adminis- l by bolus or constant infusion (Figs. 3– Thus conjugation of DCA with glycine to parenteral administration does not nce the choleretic effect. The excretion of 3α-hydroxy bile salts as measured by the 3α-hydroxysteroid assay increased in a similar manner after both DCA and GDCA administration. We found DCA to be essentially completely metabolized in these experiments as previously reported by Soloway *et al.* (7). Thus the 3α-hydroxysteroid excretion reflects reductive metabolism of DCA and GDCA to a similar extent.

Bile from dogs 3 and 4 was analyzed for 3α-hydroxy bile salts and for electrolytes for calculation of anion gaps. Any appreciable excretion of unchanged GDCA or DCA should be reflected in a difference between the 3α-hydroxy assay and anion gap. However, no significant discrepancy was found in these dogs (Figs. 4 and 5). Thus in these experiments the 3α-hydroxy assay reflects excretion of similar amounts of 3α-hydroxy-metabolites after GDCA and DCA administration. Thin-layer chromatography of bile from these two experiments showed only trace amounts of unchanged DCA and small amounts of GDCA which approximated the amount of glycocholic acid (**IVb**) in the bile.

Summary. Our results demonstrate extensive metabolism of GDCA in the dog and rat by a pathway similar to that for DCA in man (7). Assignment of reduced metabolites and relative ratios was made by tlc comparison of tritiated metabolites with known compounds. Trace bolus doses of GDCA result in complete reductive metabolism, and increasing molar doses result in the excretion of only small amounts (<5%) of unchanged GDCA in bile. Thus our *in vivo* experiments are in contrast to the absence of GDCA metabolism reported in the isolated, perfused rat liver (12).

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ıility of Polymorphic Enzyme Phenotypes in Human Tumor Cell Lines (40711)

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reds of cultured cell lines from a large of human tumors have now been ıed as experimental models of cancer The risk of cross-contamination with ɔther lines has been demonstrated by er of cases of contamination by the ell line (12–14), and by another de- ɔntamination among a group of G-6- : B lines (15–17). By analysis of po- ıic enzymes with inherited electro- : mobility variants observed in human ɔell lines can be efficiently character- that contamination can be either de- r shown not to exist (1, 12, 13, 15–20). requisite for valid results is that phe- of polymorphic enzymes remain sta- *'ro* and *in vivo*. Ideally, to demonstrate pe stability, cell strains derived from tissues and lines from malignant tu- ould be compared with each patient's and malignant cells. The logistics, r, have usually been too complicated ɔletely achieve this end. The present :ports on a partial stepwise approach a satisfactory answer to this question l provides some of the rationale and ɔn of our ongoing analysis of about nan tumor cell lines during the last years which has been reported briefly , and will be published in detail.

'ials and methods. Specimens of both .nt tumors and normal tissues corre- g to the tissue of origin of each tumor noved at biopsy or autopsy from each .tients with the following carcinomas: 3; colon; renal cortex; lung, 4; stom- ınd from one patient with Hodgkin's ma. The tissues were kept frozen until for enzyme analysis could be made ogenization with 1–3 vol of distilled ollowed by centrifugation and collec- he supernatant. Human tumors prop- n nude mice were similarly processed. ın tumor cell lines, grown as mono- r suspension cultures, and cultured skin fibroblast strains were harvested and washed with saline. Samples were lysed with 1–2 parts distilled water followed by freeze–thawing two to three times and centrifuged at 2000 rpm for 15 min at 4°C. References to data on the origin and other characteristics of the cell lines reported in this paper are given in Tables II and III. Human erythrocytes were collected from heparinized blood, the serum separated by centrifugation was discarded, and the erythrocytes were washed three times with saline.

The cell samples were stored at −80°C which was found to preserve enzyme activity and mobility patterns for months; in some cases, as an extra precaution, NADP and mercaptoethanol were added to stabilize enzyme activity and maintain enzyme electrophoretic mobility patterns. Electrophoresis was carried out according to methods of Harris and Hopkinson (22). The following enzymes were analyzed by the methods given in the references: glucose-6-phosphate dehydrogenase, G-6-PD (22–24); acid phosphatase, ACP_1 (22, 25); glyoxalase I, GLO (26); malic enzyme, mitochondrial, MEm (27–29); phosphoglucomutase, PGM_3 (30); α-fucosidase, FUC, by isoelectric focusing (31); phosphoglucomutase, PGM_1 (32); esterase D, ESD (33); adenosine deaminase ("red cell" isozyme), ADA (34); adenylate kinase, AK_1 (35); 6-phosphogluconate dehydrogenase, PGD (22, 36); glutamate–oxaloacetate transaminase, GOTm (22); and peptidases, PEPA (22, 37), PEPB (38), PEPC (39), and PEPD (40). The buffer system for GLO consisted in part of 0.03 *M* diethylbarbituric acid (MW 184.19) rather than barbituric acid (MW 128.09) as stated in Ref. (26). The peptidases are included as a polymorphic group of loci, although strictly speaking PEPA, B, or C may not quite qualify as polymorphic in the Caucasian or Negro populations using the methods indicated. The phenotypes for these enzymes are expressed with standard accepted

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terminology: homozygotes are 1, 2, A, B, or C, where the same allele occurs at the same locus on homologous chromosomes in an individual or cell line; heterozygotes are 2-1, BA, CB, or CA where two different alleles occur together.

Control samples for various enzyme phenotypes were kindly provided by Drs. Lynda J. Donald, Department of Pediatrics, University Hospital, London, Ontario, Canada (PGM_1, PGM_3); Phyllis J. McAlpine, Children's Centre, Health Science Centre, Winnipeg, Manitoba, Canada (ESD, ADA, AK_1, PGD); Susan M. Povey, MRC Human Biochemical Genetics Unit, the Galton Laboratory, University College London, England (controls for many of the enzymes); Bryan M. Turner and N. G. Beratis, Division of Medical Genetics, Department of Pediatrics, Mount Sinai School of Medicine, New York, (FUC); and Geoffrey O'Neill, Sloan-Kettering Institute for Cancer Research, New York (GLO). In addition, erythrocytes of W.C.W., typed at the Galton Laboratory, were used as a control for several enzymes. Phenotype frequency products were obtained by multiplying the frequencies of all observed phenotypes for a cell line using frequencies published for normal populations of the same race as the patient from which the particular cell line originated (22).

Results. During the stages from normal human tissue to malignant tumor to established tumor line *in vitro*, and to tumor propagated from cells of this line in nude mice, the following comparisons demonstrate that phenotypes of the polymorphic enzymes used in our characterization studies of cultured human tumor cell lines remain stable.

1. When the 12 malignant human tumors were compared with 12 corresponding normal tissues for isozyme patterns of G-6-PD, PGM_3, FUC, PGM_1, ESD, ADA, AK_1, and PGD, concordant phenotypes (57 total) were observed in all sets, showing that the conversion of normal to malignant cells in the patients had not changed the phenotypes (Table I). The data presented here are not complete for all 24 tissues, since some enzymes in certain specimens were not sufficiently active to be phenotyped. FUC 2-1 and 2 phenotypes could not be distinguished from each other in these specimens, but usually were distinguished in cultured cells.

2. A comparison of a skin fibroblast culture with red blood cells (Table II) from the same normal donor showed five concordant phenotypes out of five comparisons (G-6-PD, PGM_1, ESD, AK_1, PGD). Thus, cultivation *in vitro* had not changed these phenotypes.

3. Two cultured human tumor cell lines were compared with normal skin fibroblasts cultured from the same two patients (Table II). The kidney tumor line, Caki-1, and the related skin fibroblast strain had nine out of nine phenotypic comparisons identical for G-6-PD, PGM_3, FUC, PGM_1, ESD, AK_1, and PEPA, B, and C. The melanoma line, Malme-3M, and the fibroblast strain from the same patient had 13 identical phenotypes out of 13 enzymes including the above 9 enzymes, and including ACP_1, GLO, MEm, and PEPD.

TABLE I. IDENTICAL PHENOTYPES OF EIGHT ENZYMES FOR SETS OF MALIGNANT AND CORRESPONDING NORMAL TISSUE FROM 12 PATIENTS.

Tissue set	G-6-PD	PGM_3	FUC	PGM_1	ESD	ADA	AK_1	PGD	Freq. Product
Breast				1	1		1		0.4318
Breast		2-1		1	2-1	1	1		0.0333
Breast		1	1	2-1	1	1	1	A	0.0674
Colon	B	2-1	[a]	2-1	2-1				0.0253
Kidney		2-1	[a]	2-1	1		1		0.1052
Lung		1	[a]	1	1		1		0.2333
Lung		1	[a]	1	1		1		0.2333
Lung	B	1	1	2-1	1				0.0880
Lung	B	1		1	1		1		0.2333
Hodgkin's dis.		2	1	2-1	1		1		0.0089
Gastric		1	1	1	2-1	1		A	0.0266
Gastric		2-1	1	1	1		1		0.0970

[a] Two cathodal bands of equal intensity: probably phenotype 2-1 (frequency 0.3814), less likely phenotype 2 (frequency 0.0567).

TABLE II. IDENTICAL PHENOTYPES OF 16 ENZYMES IN ERYTHROCYTES/CULTURED SKIN FIBROBLASTS, CULTURED KIDNEY CARCINOMA LINE/CULTURED FIBROBLASTS, AND CULTURED MELANOMA LINE/CULTURED FIBROBLASTS, EACH SET DERIVED FROM SAME DONOR.

Donor diagnosis	Cell type	G-6-PD	ACP_1	GLO	MEm	PGM_3	FUC	PGM_1	ESD	ADA RBC	Tiss.	AK_1	PGD	GOTm	PEPA	PEPB	PEPC	PEPD	Freq. product	Passages tested	Reference
Normal	RBC	B			No act.	No act.		2-1	2-1			1	A						0.0548		
	Cultured skin	B			2-1	1		2-1	2-1			1	A						0.0150	7, 10, 11	
Ca. kidney	Caki-1	B	B	2-1	2	2-1	2-1	1	2-1	1	d	1	A	1	1	1	1		0.0001	14, 19, 26, 43	4
	Caki-1 skin	B				2-1	2-1	1	2-1		d	1			1	1	1	1	0.0139	14, 20	
Melanoma	Malme-3M	B	BA	2-1	1	1	1	1	1	1		1	CA	1	1	1	1	1	0.0004	10, 43, 48, 50, 51	4
	Malme-3 skin	B	BA	2-1	1	1	1	1	1		d	1			1	1	1	1	0.0112	3	

These phenotypes, therefore, were stable during tumorigenesis, as well as cultivation during many passages of both malignant and normal cells.

4. Constancy of phenotypes shown in Table III of G-6-PD, ACP_1, GLO, MEm, PGM_3, FUC, PGM_1, ESD, ADA, AK_1, PGD, GOTm, and peptidases A, B, and C was also found between lines derived from the same patient. Included were two series of tumor lines, with each series derived from a different patient: the MDA-MB-175 series of lines I, II, III, IV, VI, and VII and the MDA-MB-331 series, II and IV. Each line was derived from a pleural effusion, taken at intervals of a number of weeks. Also included in the analysis were the following eight pairs or sets of cell lines where lines in a pair were either derived from the same tumor or one line was later cultured as a subline: SK-LU-1 and SK-LU-1b, lung carcinoma; 734B and MCF-7, breast carcinoma; C-33A and C-33B, C-4 I and C-4 II, and two HeLa lines ($HeLa_1$ and $HeLa_2$) cultured separately for many years, carcinomas of the cervix; SK-UT-1 and SK-UT-1b, primary mixed mesodermal tumor of the uterus; HEC-1A and HEC-1B, adenocarcinoma of the endometrium; SK-MEL-26 monolayer and SK-MEL-26 suspension, malignant melanoma. All 95 comparisons of phenotypes within sets were concordant. The sum of the numbers of passages of different lines within sets (for example, HEC-1A and HEC-1B, 117 + 122 = 239 passages) attests to the high genetic stability of these phenotypes during the course of separate culture.

5. A comparison of two previously unpublished lines established from the same carcinoma specimen (probably lung) in this laboratory, Calu-6 as a cultured cell line and PR 164 as a tumor line in nude mice, showed identical phenotypes for MEm 1 (frequency = 0.44) and PGM_3 2 (frequency = 0.06). These human isozymes, therefore, were stable in human tumor cells grown in parallel *in vitro* and in the nude mouse.

Discussion. We are not aware of previous extensive comparisons of polymorphic enzyme phenotypes between human tumor cell lines and the normal or malignant tissues of the patients of origin. In the present study, many enzymes were shown to have stable electrophoretic phenotypes in all the *in vivo*

TABLE III. IDENTICAL PHENOTYPES OF 15 ENZYMES IN 10 SETS OF CULTURED HUMAN TUMOR CELL LINES, EACH SET DERIVED FROM ONE PATIENT.

Tissue	Patient no.	Cell line	G-6-PD	ACP_1	GLO	MEm	PGM_3	FUC	PGM_1	ESD	ADA	AK_1	PGD	GOTm	PEPA	PEPB	PEPC	Freq. product	Passages tested	References
Lung	1	SK-LU-1	B		2	1	1	2	2	2		1						2.0×10^{-6}	35, 48	4
		SK-LU-1b	B				1	2	2	2	1	1	A		1	1	1	1.2×10^{-5}	19	
Breast	2	734 B	B	CB	2-1		1	1	2-1	2-1	1				1	1	1	0.0005	6 (ours)	41
		MCF-7	B	CB	2-1		1	1	2-1	2-1	1	1	A	1	1	1	1	0.0004	22, 31	41
	3	MDA-MB-175 I	B				2-1		2	1		1						0.0049	10, 25	4
		MDA-MB-175 II	B				2-1		2	1								0.0051	45, 50	4
		MDA-MB-175 III	B		2						1				1	1	1	0.1322	42	4
		MDA-MB-175 IV	B				2-1		2	1								0.0050	16, 40	4
		MDA-MB-175 VI	B				2-1		2	1								0.0050	5, 18	4
		MDA-MB-175 VII	B				2-1		2	1								0.0050	20, 38, 41, 45	4
	4	MDA-MB-331 II	B			2	1		1	1		1						0.0124	5	
		MDA-MB-331 IV	B				1		1	1		1						0.2333	5	
Cervix	5	C-33A	B	BA	2	2	1	1	1	1	1	1	A	1	1	1	1	0.0007	89, 93	42, 43
		C-33B	B	BA		2	1		1	1	1	1	A	1	1	1	1	0.0041	86, 92	42, 43
	6	C-4 I	B	B	1	1	1	2	1	1	1	1	A	2-1				8.6×10^{-6}	86	42, 43
		C-4 II	B		1	1	1	2	1	1	1	1	A	2-1				2.2×10^{-5}	91	42
	7	$HeLa_1$	A	BA	2	2-1	1	1	1	1	1	1	A		1	1	1	0.0002		44
		$HeLa_2$	A	BA	2	2-1	1	1	1	1	1	1	A		1	1	1	0.0002		45
Uterus	8	SK-UT-1	B	B	2-1	2-1	1	2-1	1	1	1	1	A					0.0073	3, 30	4
		SK-UT-1b	B	B	2-1		1	2-1	1	1	1	1	A	1				0.0144	29	
	9	HEC-1A	B	BA	2	1	2-1	1	1	1	1	1	A	1					117	46, 47
		HEC-1B	B	BA	2		2-1	1	1	1	1	1	A	1					122	46
Melanoma	10	SK-MEL-26[a]	B	BA	2-1	1	1	1	2-1	2-1	1	1	A	1				0.0013	8, 9, 21, 69	9
		SK-MEL-26[b]	B	BA		1	1	1	2-1	2-1	1			1				0.0029	70	9

[a] Monolayer.
[b] Suspension.

n vitro situations compared. Paired sam- of tumor and normal tissues from 100 nts have previously been reported to concordant G-6-PD and PGM_1 pheno- (13). However, our study also includes $[_3$, FUC, ESD, ADA, AK_1, and PGD in d samples from 12 patients. Several fi- ast strains derived from normal persons were typed directly for G-6-PD and $[_1$ were found to maintain the enzyme otypes in culture (13). Our study con- this comparison and extends it to in- ESD, AK_1, and PGD. Stability has also reported for phenotypes of G-6-PD and $[_1$ in cell strains after cultivation for 3 , after infection with mycoplasma and), after treatment with drugs (13), and X irradiation (13, 48). In our analysis of umor lines and normal fibroblast strains the same patients, all phenotypes within r were concordant. Stability of isozyme lities has been observed between normal transformed cells. For example, estab- l cell lines of the Chinese and Syrian ters and the mouse maintained the same es-specific isozymes as seen in fresh tis- (49). Furthermore, when fresh erythro- of human donors were compared with hoblastoid lines derived from their leu- es stability of phenotypes was observed

ng-term cultivation of human tumor separated by as many as 239 passages, ot change the phenotypes, thus confirm- reviously observed stability over 3 years *vitro* cultivation for two human tumor (13). Likewise, phenotype stability after term culture was reported for 26 en- s in 66 human lymphoblastoid lines with two exceptional changes, from hetero- ity to homozygosity, observed in two grown for many years in several labo- ies (18). However, in the present study terozygous phenotypes in tissue samples ll lines were observed with complete ordance with the heterozygous pheno- observed in other material or cell lines the same patient.

e observation that MEm and PGM_3 otypes were the same in a cultured line nude mouse-grown tumor line derived the same human tumor agrees with ous reports that growth of human tumor cells in the nude mouse does not affect the electrophoretic mobilities of several other enzymes (50, 51). Similar results have been observed for tumors in nude mice originating from cell lines of the Chinese and Syrian hamsters, mouse, rat, rabbit, and man, and with human–rodent cell hybrids (52–56).

An interesting example of phenotype stability, which might have been considered as a case of instability, was reported for a group of black female patients who were G-6-PD type BA (57–60). Benign or malignant tumors and tumor cell lines from these patients were either A or B, not BA. Taking into account that G-6-PD is X linked and subject to the Lyon effect, the explanation given was that these tumors arose as single cell clones. This problem would not occur for other enzymes studied presently since they are not X linked.

The stability of polymorphic enzyme phenotypes contrasts with the relative instability of chromosomes in tumors (61) and cultured cell lines (62). Because of variation of karyotypes within a cell line karyotypic variation occurs between sublines or clones established from a line. Similarly, since cases of cross-contamination are likely due to the transfer of only a few or even single cells, a cross-contaminated line may have a karyotype different from that of the contaminating lines. This interpretation can be made from the karyotypic analysis of a number of human tumor cell lines determined by polymorphic enzyme analysis to be a contamination group (17).

Whether the demonstration of a few identical marker chromosomes in two or more cell lines carries sufficient weight to declare them as cross-contaminated has become more problematical as a consequence of recent karyotypic studies of human tumors and tumor cell lines with chromosome banding techniques. These studies have shown that specific translocations and other karyotypic modifications involving a nonrandom selection of chromosomes occur frequently and can be correlated with specific types of cancer (63–70). Therefore, tumor cell lines derived from tumors of different patients with certain types of cancer could be expected, in some cases, to have certain similar marker chromosomes. Also, marker chromosomes resembling the HeLa markers were detected in a

pleural effusion of a black female patient with breast cancer and in a cell line derived from her pleural effusions (71). Such a cell line might have been considered as HeLa cell contaminated if the patient's own effusions had not been examined directly. Furthermore, the results of analysis of 15 polymorphic enzymes showed that two enzymes could distinguish the patient and the cell line from HeLa cells. Therefore, because of (a) the lack of frequency data on the occurrence of the independent origin of identical marker chromosomes in particular types of tumors and derived cell lines and (b) the inconsistent presence of markers in all cells, clones, or sublines of a line, one cannot give a statistical test of significance to evaluate karyotypic data as can be done with polymorphic enzyme data.

The overwhelming concordance of phenotypes in the various comparisons made in this study of many polymorphic enzymes on human tumor cells confirms and extends other reports on stability. This signifies that these enzyme phenotypes are stable in spite of cellular changes to malignant or transformed status and on long-term tissue culture. Therefore, these enzymes can be effectively used to individually distinguish human tumor cell lines, and the phenotype frequency data obtained from normal human populations are applicable for the calculation of the frequency of the combination of phenotypes of a cell lines.

Summary. Concordance of polymorphic enzyme phenotypes in various comparisons of human normal and malignant cells *in vitro* and *in vivo* establishes isozyme stability after change to the malignant state and after long-term tissue culture. Such enzymes, therefore, can be used with considerable confidence to individually distinguish human tumor cell lines. The stability validates the use of phenotype frequency data obtained from surveys of normal human populations in the analysis of data on human tumor cell lines.

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